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(54) **Novel polypeptide.**

(57) Novel hG-CSF polypeptide derivatives having an amino acid sequence derived from the amino acid sequence of the human granulocyte colony stimulating factor polypeptide by substitution of at least one amino acid by a different aminoacid and/or deletion of at least one amino acid, recombinant plasmids containing a DNA fragment insert coding for any of these hG-CSF polypeptide derivatives, microorganisms carrying one of such plasmids, and methods of producing the hG-CSF polypeptide derivatives using the microorganisms are described.

NOVEL POLYPEPTIDE

FIELD OF THE INVENTION

The present invention relates to novel human granulocyte colony stimalating factor (hG-CSF) polypeptide derivatives, recombinant plasmids with a DNA coding for any of said polypeptide derivatives being inserted therein, microorganisms each carrying any of said plasmids, and a method of producing said novel hG-CSF polypeptide derivatives.

BACKGROUND OF THE INVENTION

The human granulocyte colony stimulating factor (hG-CSF) is a kind of polypeptide which is essential in the formation of various blood cells as a result of proliferation and differentiation of hematopoietic stem cells. Its major effect is to promote the increase in number of granulocytes, in particular neutrophils. Neutrophils play an important part in the protection of the living body from infection.

However, their life spans are short and, therefore, constant supplementation is required by proliferation and differentiation of precursor cells. The therapies widely employed in recent years for proliferative tumors simultaneously inhibit the growth of neutrophil precursors, hence cause a severe side effect, namely a reduction in neutrophilic protection in cancer-bearing patients making them more susceptible to infection. hG-CSF is expected to be effective in alleviating this undesirable side effect through promotion of the increase in the number of neutrophils on one hand and, on the other, in preventing and treating infectious diseases. Furthermore, hG-CSF is active in causing differentiation of leukemic cell lines in vitro and therefore may possibly be useful as a therapeutic agent for leukemia. The hG-CSF polypeptide derivatives according to the invention are superior in hG-CSF activity to the known hG-CSF and are expected to be useful as drugs.

With the recent rapid progress in recombinant DNA technology, genes for proteins involved in the proliferation and differentiation of blood cells have been isolated in succession. Such factors are in production by genetic engineering techniques using microorganisms or animal cells.

A cDNA for hG-CSF was isolated from the human squamous cell carcinoma cell line CHU-II, its base sequence determined and its expression in COS cells reported by Nagata et al. [Nagata et al.: Nature, 319, 415 (1986)]. Souza et al. also isolated a cDNA from the human bladder cancer cell line 5637, determined its base sequence and reported its expression in Escherichia coli (E. coli) [Souza et al.: Science, 232, 61 (1986)].

The amino acid sequence of the protein encoded by the above two cDNAs is in agreement with the amino acid sequence (Table 1) of the protein encoded by the cDNA isolated from normal human peripheral blood macrophages by the present inventors.

Table 1

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1       10        20        30        40        50
ACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCAAGTGCTTAGAG
ThrProLeuGlyProAlaSerSerLeuProGlnSerPheLeuLeuLysCysLeuGlu
1
 60        70        80        90        100       110
CAAGTGAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAGGAGAAGCTGTGTGCCACC
GlnValArgLysIleGlnGlyAspGlyAlaAlaLeuGlnGluLysLeuCysAlaThr

  120       130       140       150       160       170
TACAAGCTGTGCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCATCCCC
TyrLysLeuCysHisProGluGluLeuValLeuLeuGlyHisSerLeuGlyIlePro

      180       190       200       210       220
TGGGCTCCCCTGAGCAGCTGCCCCAGCCAGGCCCTGCAGCTGGCAGGCTGCTTG
TrpAlaProLeuSerSerCysProSerGlnAlaLeuGlnLeuAlaGlyCysLeu

  230       240       250       260       270       280
AGCCAACTCCATAGCGGCCTTTTCCTCTACCAGGGGCTCCTGCAGGCCCTGGAAGGG
SerGlnLeuHisSerGlyLeuPheLeuTyrGlnGlyLeuLeuGlnAlaLeuGluGly

     290       300       310       320       330
ATCTCCCCCGAGTTGGGTCCCACCTTGGACACACTGCAGCTGGACGTCGCCGAC
IleSerProGluLeuGlyProThrLeuAspThrLeuGlnLeuAspValAlaAsp

 340       350       360       370       380       390
TTTGCCACCACCATCTGGCAGCAGATGGAAGAACTGGGAATGGCCCCTGCCCTGCAG
PheAlaThrThrIleTrpGlnGlnMetGluGluLeuGlyMetAlaProAlaLeuGln

    400       410       420       430       440       450
CCCACCCAGGGTGCCATGCCGGCCTTCGCCTCTGCTTTCCAGCGCCGGGCAGGAGGG
ProThrGlnGlyAlaMetProAlaPheAlaSerAlaPheGlnArgArgAlaGlyGly

       460       470       480       490       500
GTCCTAGTTGCCTCCCATCTGCAGAGCTTCCTGGAGGTGTCGTACCGCGTTCTACGC
ValLeuValAlaSerHisLeuGlnSerPheLeuGluValSerTyrArgValLeuArg

510       520
CACCTTGCCCAGCCCTGA
HisLeuAlaGlnPro***
      174
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SUMMARY OF THE INVENTION

It is an object of the invention to provide a means of producing, at low cost and in large quantities, hG-CSF polypeptide derivatives having high specific activity and high stability in blood.

The present inventors found that hG-CSF polypeptide derivatives having high specific activity can be produced by modifying the cDNA for hG-CSF shown in Table 1 and cultivating a strain of E. coli that harbors a plasmid with the modified cDNA inserted therein or by limited polypeptide decomposition using a protease, and they have now completed the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a construction scheme for the plasmid pCfTA1.

Fig. 2 shows a construction scheme for the plasmid pCfTB20.

Fig. 3 shows construction schemes for the plasmids pCfTL23, 38, 35 and 41.

Fig. 4 shows construction schemes for the plasmids pCfTM14, 17 and 113.

Fig. 5 shows a construction scheme for the plasmid pCfWD1.

Fig. 6 shows construction schemes for the plasmids pCfT95K19, pCfAA1 and pCfAB5.

Fig. 7 shows construction schemes for the plasmids pCfBA3, pCfBB101, pCfBC52, 59, 42B1, 45, 76, 77, 93, 95, 97, pCfBD28, 56 and 82.

Fig. 8 shows construction schemes for the plasmids pCfCB101, pCfCC52, 59, pCFCD28 and 56.

Fig. 9 (1) and (2) schematically show the processes involved in the Okayama-Berg method for cDNA synthesis and construction of a recombinant plasmid containing the DNA synthesized.

Fig. 10 shows a construction scheme for the plasmid pLA1.

Fig. 11 shows a construction scheme for the plasmid pCfTNS501.

Fig. 13 shows a construction scheme for the plasmid pTrS20.

Fig. 14 shows construction schemes for the plasmids pCfTNS7 and pCfTAArg4S.

Fig. 15 shows a construction scheme for the plasmid pCfTN205 and pCfTAArg4.

Fig. 16 shows construction schemes for the plasmids pCfTNS301 and pCfTNS401.

Fig. 17 (1) and (2) show construction schemes for the plasmids pCfBD28A17 and pCfBD28T17.

DETAILED DESCRIPTION OF THE INVENTION

The hG-CSF polypeptide derivatives according to the invention differ in part of the amino acid sequence from the hG-CSF polypeptide having the amino acid sequence shown in Table 1 as a result of substitution and/or deletion. The amino acid or amino acids to be substituted are those amino acids that are located at or in the neighborhood of the N terminus. Preferably, at least one amino acid from among the 1st to 17th amino acids from the N terminus should be the target of substitution. Similarly, the amino acids to be deleted are those amino acids at or in the neighborhood of the N terminus. Preferably, at least one amino acid from among the 1st to IIth amino acids from the N terminus should be deleted.

The recombinant plasmids according to the invention are obtained by inserting a DNA fragment coding for any of the above mentioned hG-CSF polypeptide derivatives into an appropriate plasmid having a DNA expression function.

Preferred as the DNA fragments coding for the hG-CSF polypeptide derivatives of the invention are those resulting from substitution of at least one base selected from among the 1st to 51st bases of the base sequence shown in Table 1 of the DNA coding for hG-CSF.

A cDNA (hG-CSF cDNA) obtained by reverse transcription of an hG-CSF-encoding messenger RNA by recombinant DNA technology or an hG-CSF-encoding DNA obtained from chromosomal DNA, for instance, can be used as the hG-CSF-encoding DNA shown in Fig. 1.

Any hG-CSF cDNA may be used provided that it codes for hG-CSF. As an specific example, pCSF1-2, a plasmid produced by the present inventors, can be used. The process for the production of pCSF1-2 is described in Reference Example 1.

The hG-CSF cDNA contained in pCSF1-2 has the base sequence shown in Table 1 as determined by the dideoxy sequencing method using M13 phage [J. Messing et al.: Gene, 19, 269 (1982)].

pCSF1-2 is a plasmid having the restriction enzyme map shown in Fig. 1 and an E. coli strain containing it, E. coli ECSF 1-2, has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology (FRI) since November 27, 1986 under the deposit number FERM BP-1220 in accordance with the Budapest treaty.

Any plasmid may be used for the insertion of an hG-CSF polypeptide derivative-encoding DNA thereinto provided that said DNA can be expressed in E. coli.

A plasmid can be used with advantage which allows foreign DNA insertion thereinto at a site downstream from an appropriate promoter, for example, a trp or lac promoter, and has the distance between the Shine-Dalgarono sequence (hereinafter SD sequence) and the initiation codon (ATG) adjusted to an appropriate distance, for example, 6-18 base pairs.

As suitable examples of such plasmid, there may be mentioned pKYP10 (US Patent 4,686,191) pLSA1 (Reference Example 3), pGEL1 [Sekine et al.: Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)], pKYP26 [Japanese Patent Application (OPI) No 48 699/87 (the term "OPI" means an unexamined published

application) and pBR322 (Bolivar et al.: Gene, 2, 95 (1977)].

Recombination between a DNA coding for the hG-CSF polypeptide or a derivative thereof and a vector DNA can be effected by recombinant DNA techniques in general use which comprise digesting both DNA with a restriction enzyme or enzymes and the subsequent ligation using T4 DNA ligase. For ligation, the DNA fragment termini resulting from restriction enzyme digestion may be processed, when appropriate, by making use of the filling-in reaction using DNA polymerase I Klenow fragment or the trimming reaction using T4 DNA polymerase; the DNA linker technique is also applicable.

Examples of the construction of recombinant plasmids containing an hG-CSF polypeptide derivative-encoding DNA inserted therein by using pCSF1-2 as the hG-CSF cDNA, pGEL1, pKYP10, pKYP26, pBR322 or pLSA1 as the plasmid for incorporation of the DNA thereinto and, as necessary, a chemically synthesized DNA linker or a technique of site-specific mutagenesis are given in the following

DETAILED DESCRIPTION OF THE DRAWINGS

(Recomnination Procedures)

As shown in Fig. 1, pCSF1-2 [about 4.5 kilobases (hereinafter kb)] is cleaved with ApaI and Bam HI and a DNA fragment of about 1.5 kb is purified by low gelling temperature agarose gel electrophoresis (LGT method) [L. Wieslander: Analytical Biochemistry, 98, 305 (1979)].

Then, pLSA1 is cleaved with BanIII and BamHI, and a DNA fragment of about 2.8 kb is purified by the LGT method. Both the fragments thus obtained and the synthetic DNA shown in Fig. 1 are ligated together using T4 DNA ligase to give pCfTA1.

Then, as shown in Fig. 2, pCfTA1 is cleaved with BamHI, the protruding ends are converted to blunt ends by treatment with the Klenow fragment and, after further cleavage with Eco RI, a DNA fragment of about 2.5 kb is purified by the LGT method. Separately, pCfTA1 is cleaved with EcoRI and DraI and a DNA fragment of about 1.0 kb is purified by the LGT method. The DNA fragments thus obtained are ligated together using T4 DNA ligase to give pCfTB20.

Further, as shown in Fig. 3, pCSF1-2 is cleaved with ApaI and BamHI and a DNA fragment of about 1.5 kb is purified by the LGT method. Separately, pGEL1 is cleaved with HindIII, BamHI and PstI and a DNA fragment of about 1.7 kb is purified by the LGT method. Furthermore, pKYP10 is cleaved with PstI and BanIII and a DNA fragment of about 1.1 kb is purified by the LGT method. Ligation of these three DNA fragments and the synthetic DNA shown in Fig. 3 gives pCfTL23, pCfTL38, pCfTL35 and pCfTL41 whereas ligation of these three DNA fragments and the synthetic DNA shown in Fig. 4 gives pCfTM14, pCfTM17 and pCfTM113.

Furthermore, as shown in Fig. 5, pCfTA1 is cleaved with BanIII and StuI and an hG-CSF cDNA-containing DNA fragment of about 1.3 kb is purified by the LGT method. Separately, pKY26 is cleaved with BamHI, the protruding ends are converted to blunt ends by treatment with DNA polymerase I Klenow fragment and, after further cleavage with PstI, a DNA of about 1.8 kb is purified by the LGT method. Further, separately, pGEL1 is cleaved with BanIII and PstI and a DNA fragment of about 1.0 kb is purified by the LGT method. The three DNA fragments thus obtained are ligated together using T4 DNA ligase to give pCfWD1.

Further, as shown in Fig. 6, pCfTL38 is cleaved with HindIII and BglII and a DNA fragment of about 2.6 kb is purified by the LGT method. Separately, pCfTL38 is cleaved with HindIII, BamHI and DpnI and a DNA fragment of about 300 bp (base pairs) is purified by the LGT method. Further, separately, pCfTB20 is cleaved with AvaI, the protruding ends are pared off by treatment with the Klenow fragment and, after further cleavage with BglII, a DNA fragment of about 480 bp is purified by the LGT method. The three DNA fragments thus obtained are ligated together using T4 DNA ligase to give pCfT95K19. Further, as also shown in Fig. 6, pCfT95K19 is cleaved with BanIII and BglI and a DNA of about 1.0 kb is purified by the LGT method and, separately, pCfT95K19 is cleaved with BglI alone and a DNA fragment of about 1.8 kb is purified by said method. Further, separately, pCfT95K19 is cleaved with BglI and Sau3A and a DNA fragment of about 350 bp is purified by the LGT method. The three DNA fragments thus obtained and the synthetic DNA shown in Fig. 6 in the middle thereof (i.e. halfway down) are ligated together to give pCfAA1. Then, as also shown in Fig. 6, pCfAA1 is cleaved with Xho I and BglI and a DNA fragment of about 3.0 kb is purified by the LGT method. This fragment, the above-mentioned BgI I-Sau3A fragment (about 350 bp) of pCfT95K19 and the synthetic DNA shown in Fig. 6 at the bottom thereof are ligated together using T4 DNA ligase to give pCfAB5 and pCfAB14. Further, as shown in Fig. 7, pCfAB5 is cleaved with AvaI and BgI II and a DNA fragment of about 2.8 kb is purified by the LGT method. Separately, pCfWD1 is cleaved with BglII

and AvaI and the DNA of about 1.3 kb is purified by the LGT method. The two fragments thus obtained are ligated together using T4 DNA ligase to give pCfBA8. On the other hand, pCfAB14 is cleaved with AvaI and BglII and a DNA fragment of about 2.8 kb is purified by the LGT method, and this fragment is ligated with the above-mentioned 1.3 kb DNA fragment derived from pCfWD1 by cleavage with BglII and AvaI using T4 DNA ligase, to give pCfBA32. Further, as also shown in Fig. 7, pCfBA8 is cleaved with BanIII, BglII and XhoI and a DNA fragment of about 1.4 kb and a DNA fragment of about 2.7 kb are purified by the LGT method. Ligation of the two DNA fragments thus obtained and the synthetic DNA linker shown inFig. 7 using T4 DNA ligase gives pCfBB101, pCfBC52, pCfBC59, pCfBD28, pCfBD56, pCfBC42B1, pCfBC45, pCfBC76, pCfBC77, pCfBC93, pCfBC95, pCfBC97 and pCfBD82.

As shown in Fig. 8, pBR322 is cleaved with PstI, the protruding ends are pared off with T4 DNA polymerase, the BglII linker is inserted using T4 DNA ligase and, after further cleavage with EcoRI and BglII, a DNA fragment of about 3.6 kb is purified by the LGT method.

The plasmids pCfBB101, pCfBC52, pCfBC59, pCfBD28 and pCfBD56 obtained in the above manner are each cleaved with EcoRI and BglII and a DNA fragment of about 1.8 kb is purified by the LGT method. Each 1.8 kb DNA fragment is ligated with the pBR322-derived 3.6 kb DNA fragment using T4 DNA ligase. There are thus obtained pCfCB101, pCfCC52, pCfCC59, pCfCD28 and pCfCD56 corresponding to the respective plasmids mentioned above.

On the other hand, pCfBA8 is cleaved with Ban III, BglII and and a DNA fragment of about 2.7 kb is purified by the LGT method. Separately, pCfBA8 is cleaved with Xho I and BglII and a DNA fragment of about 1.4 kb is purified by the LGT method. Ligation of the two fragments thus obtained and the synthetic DNA linker shown in Fig. 14 gives pCfTNS7 and pCfTAArg4S. In addition, as shown in Fig. 15, pCfTNS7 is cleaved with PvuI and XhoI and a DNA fragment of about 1 kb is purified by the LGT method. Separately, pCfBA32 is cleaved with PvuI and Xho I and a DNA fragment of about 3 kb is purified by the LGT method. The two fragments thus obtained are ligated together using T4 DNA ligase to give pCfTN205. Similarly, pCfTAArg4S is cleaved with PvuI and XhoI, a fragment of about 1 kb is purified by the LGT method and this fragment is ligated with a DNA fragment of about 3 kb derived from the above-mentioned plasmid pCfBA32 by cleavage with PvuI and Xho1, using T4 DNA ligase to give pCfTAArg4. Furhter, pCfBA8 is cleaved with BanIII and BglII and a DNA fragment of about 2.7 kb is purified by the LGT method. Separately, pCfBA8 is cleaved with XhoI and BglII and a DNA fragment of about 1.4 kb is purified by the LGT method. The two fragments thus-obtained and the synthetic linker shown in Fig. 16 are ligated together using T4 DNA ligase to give pCfTNS301 and pCfTNS401. Furthermore, as shown in Fig. 12, pCfBA8 is cleaved with XhoI, the protruding ends are converted to blunt ends by Klenow fragment treatment and, after further cleavage with Pvu I, a DNA fragment of about 3 kb is purified by the LGT method. Separately, the ATG vector pTrS20 (reference Example 4) is cleaved with SacI, followed by conversion of the protruding ends to blunt ends by Klenow fragment treatment. After further cleavage with PvuI, a DNA fragment of about 1 kb is purified by the LGT method. The thus-obtained two fragments are ligated together using T4 DNA ligase to give pCfTNS501.

In an example where site-specific mutagenesis is utilized, pCfBD28 is cleaved with BanIII and PstI, as shown in Fig. 17, and a DNA fragment of about 210 bp is purified by the LGT method. Separately, the M13 phage vector M13mp19RF DNA is cleaved with AccI and PstI and a DNA fragment of about 7.24 kb is purified by the LGT method. The thus-obtained two DNA fragments are ligated together using T4 DNA ligase to give pt19BD28N. Then, this pt19BD28N is used to transfect E. coli JM105, and single-stranded pt19BD28N is recovered from the phase obtained. Similarly, as also shown in Fig. 17, the M13mp19RF DNA is cleaved with HindIII and EcoRI and a DNA fragment of about 7.2 kb is purified by the LGT method. After this 7.2 kb DNA fragment is mixed with the single-strand pt19BD28N obtained in the above manner, gapped duplex DNA formation is caused by denaturation treatment followed by annealing and the resultant gapped duplex DNA is purified by the LGT method. Then, this DNA is annealed with the synthetic DNA shown in Fig. 17 and thereafter circularized using the Klenow fragment and T4 DNA ligase. This circularized DNA is used to transfect E. coli JM105, whereby pt19BD28NA17 and pt19BD28NT17 with site-specific mutagenesis introduced therein are obtained. Further, as also shown in Fig. 17, pt19BD28NA17 and pt19BD27NT17 are cleaved with AvaI and XhoI and each DNA fragment of about 110 bp is purified by the LGT method. Separately, pCfBD28 is cleaved with Xho I and BglII and a DNA fragment of about 2.74 kb is purified by the LGT method. Further, separately, pCfBD28 is cleaved with BglII and AvaI and a DNA fragment of about 1.29 kb is purified by the LGT method. Ligation of the thus-obtained DNA fragments of about 110 bp, about 2.74 kb and 1.29 kb using T4 DNA ligase gives pCfBD28A17 and pCfBD28T17, respectivey.

The reaction conditions of the above recombination procedures are generally as follows:

The DNA digestion reaction in the presence of a restriction enzyme or enzymes is generally carried out

using 0.1-20 μg of DNA in a reaction medium containing 2-200 mM (preferably 10-40 mM) Tris-HCl (pH 6.0-9.5, preferably 7.8-8.0), 0-200 mM NaCl and 2-20 mM (preferably 5-10 mM) $MgCl_2$ at 20-70°C (the optimal temperature varying depending on the restriction enzyme(s) used) for 15 minutes to 24 hours. The restriction enzymes are each used in an amount of 0.1-100 units (preferably 1-3 units) per microgram of DNA. Termination of the reaction is generally effected by heating at 55-75°C for 5-30 minutes. It is also possible to inactivate the restriction enzymes with a reagent such as phenol or diethyl pyrocarbonate.

The DNA fragments formed by the restriction enzyme digestion or the gapped duplex DNAs can be purified by the LGT method or by polyacrylamide gel electrophoresis [A. M. Maxam et al.: Proc. Natl. Acad. Sci. USA, 74, 560 (1977)], among others.

The DNA fragment ligation reaction is carried out in a reaction medium containing 2-200 mM (preferably 10-40 mM) Tris-HCl (pH 6.1-9.5, preferably 7.8-8.0), 2-20 mM (preferably 5-10 mM) $MgCl_2$, 0.1-10 mM (preferably 0.5-2.0 mM) ATP and 1-50 mM (preferably 5-10 mM) dithiothreitol at 1-37°C (preferably 3-20°C) for 15 minutes to 72 hours (preferably 2-20 hours), using 0.3-10 units of T4 DNA ligase.

The recombinant plasmid DNA obtained by ligation reaction may be introduced into E. coli according to the transformation method of Cohen el al [S.N. Cohen et al: Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], if desired.

The recombinant M13 phage RF DNAs formed by the ligation reaction are introduced into E. coli JM105 [J. Messing et al.: Gene, 33, 103 (1985)], using the known method of transfection [Yoshiyuki Kuchino et al.: Tanpakushitsu, Kakusan, Koso (Protein, Nucleic Acid and Enzyme), 29, 294 (1984)], as necessary.

The recombinant plasmid DNAs and recombinant M13 phage RF DNAs can be isolated from the respective E. coli transformants by the method of Birnboim et al. [H.C. Birnboim et al.: Nucleic Acids Res., 7, 1513 (1973)], for example.

The isolation of the single-strand DNA from the recombinant M13 phage is carried out by the known method [Yoshiyuki Kuchino et al.: Tanpakushitsu, Kakusan, Koso, 29, 294 (1984)].

The plasmid DNAs are examined for cleavage sites by agarose gel electrophoresis or polyacrylamide gel electrophoresis following cleavage with 1-10 restriction enzymes. Further DNA base sequence determination is performed, if necessary, by the dideoxy sequencing method using M13 phase [J. Messing et al.: Gene, 19, 269 (1982)].

The desired recombinant plasmid DNAs can be produced under the conditions such as mentioned above.

The hG-CSF polypeptide derivatives of the invention can be produced in the following manner.

Thus, E. coli K-12 HB101 is transformed with a suitable plasmid (e.g. pCfBD28), and a plasmid (e.g. pCfBD28)-carrying transformant of E. coli is selected from among ampicillin resistant (hereinafter, Ap^r) colonies. Growing the plasmid (e.g. pCfBD28)-bearing strain of E. coli in a medium can lead to formation of an hG-CSF polypeptide derivative in the culture.

Any medium, whether synthetic or natural, may be used provided that it is suited for the growth of E. coli and for the production of the hG-CSF polypeptide derivative.

Usable carbon sources include glucose, fructose, lactose, glycerol, mannitol and sorbitol, among others, and usable nitrogen sources are NH_4Cl, $(NH_4)_2SO_4$, casamino acids, yeast extract, polypeptone, meat extract, Bactotryptone, corn steep liquor, etc. K_2HPO_4, KH_2PO_4, NaCl, $MgSO_4$, vitamin B_1, $MgCl_2$ and so forth may be used as other nutrient sources. The cultivation is carried out with aeration and stirring at a pH of 5.5-8.5 and a temperature of 18-40°C. Cultivation for 5-90 hours leads to accumulation of an hG-CSF polypeptide derivative in cultured cells. The cells are then harvested from the culture and disrupted by ultra sonication. Centrifugation gives cell residues. The hG-CSF polypeptide derivative is extracted from the cell residues, purified, solubilized and regenerated by the method of Marston et al. [F. A. O. Marston et al.: BIO/TECHNOLOGY, 2, 800 (1984)]. Mouse bone marrow cells are treated with said derivative, and the hG-CSF polypeptide derivative is assayed by the method using the number of colonies formed in soft agar as an index.

In the practice of the invention, the hG-CSF activity is determined in the following manner. Bone marrow cells are aseptically collected from the femur of male C3H/He mice of 8-12 weeks of age (Shizuoka Laboratory Animal Center) and suspended in α-Minimum Essential Medium (Flow Laboratories; hereinafter referred to as α-MEM) supplemented with 10% of fetal bovine serum (FBS). Nylon wool (0.3 g; Wako Pure Chemical Industries' Nylon Fiber 146-04231) packed in a column is impregnated with 1.5 ml of the above cell suspension (about 5×10^7 cells), and the reaction is allowed to proceed in a 5% CO_2 incubator at 37°C for 90 minutes. Then, α-MEM warmed to 37°C in advance is passed through the column, and bone marrow cells unadsorbed on the nylon wool are collected as an effluent fraction. The cells are washed once with α-MEM and the cell concentration is adjusted to a predetermined one.

Thereafter, the myelopoietic stem cell colony-forming ability is determined by the method of Okabe at

al. [T. Okabe, et al.: Cancer Research, 44, 4503-4506 (1986)]. Thus, 0.2 ml of the bone marrow cell suspension (2×10^6 cells/ml) prepared in the above manner is admixed with a mixture of 0.2 ml of α-MEM, 0.4 ml of FBS and 0.2 ml of each 2-fold disluted sample. An equal volume (1.0 ml) of 0.6% agar (Difco, Agar purified 0506-01) solution maintained at 42°C is admixed with the above mixute, and the resulting mixture is distributed in 0.5-ml portions onto a 24-well microtiter plate (Nunc' Multidisnh #143982) (5×10^4 cells/well, n = 3). After 7 days of incubation at 37°C in a 5% CO_2 incubator, colonies comprising not less than 40 cells are counted under a microscope (Olympus X40). After counting, each colony is transferred onto a slide glass with care, fixed there with an acetone-formalin mixed solution for 30 minutes and subjected to esterase double stain by the method of Kubota et al. [K. Kubota, et al.: Exp. Mematology, 8, 339-344 (1980)] for indentification of the colony.

The potency of each sample is calculated based on the result of counting in the colony formation test for the 2-fold dilution as follows. the activity giving half of the maximum colony formation value obtained with intact G-CSF used as a standard is defined as 50 units. The potency (in units) is calculated according to this definition and using the factor 20 for multiplication for conversion to the activity per milliliter also in view of the dilution factor for the sample. The specific activity is expressed in terms of potency (units/mg) per unit weight (mg) of protein.

The hG-CSF polypeptide derivatives lacking one or more amino acids on the N-terminal side of the hG-CSF polypeptide can also be produced by enzymatic degradation.

The derivatives can, of course, be produced by enzymatic degradation of natural hG-CSF as the starting material. However, since natural hG-CSF is low in reactivity with the enzyme (protease), the use of a modified hG-CSF having increased reactivity against protease is preferable for producing such derivatives having high activity in good yields.

Preferably used as such starting materials are the modified hG-CSFs (a), (b), (c) and (d) shown in Table 2 as resulting from substitution of one or more amino acids on the N-terminal side of the hG-CSF polypeptide. Modifications (a), (b), (c) and (d) can be obtained by cultivating bacterial strains harboring the plasmids having the corresponding base sequences, namely pCfBC59 (NC59), pCfBD28 (ND28), pCfBC95 (NC95) and pCfTAArg4S (Arg 4S), respectively, followed by isolation and purification by known methods.

Suitably used as the enzyme are endoproteases such as serine protease and thiol protease. More specifically, there may be mentioned, for example, subtilisin A, subtilisin BPN', subtilisin Carlsberg, subtilisin novo, proteinase K, nagase, thermolysin, endoproteinase Arg-C, trypsin and α-chymotrypsin. The enzyme is used is an amount of 3.4×10^6 to 8.5×10^3 units per milligram of the starting material.

Table 2

Examples of N-terminally protease-susceptible hG-CSF derivatives

Modified hG-CSF	Substituent amino acids	Plasmid*
a	Tyr^1, IIe^3, Arg^4, Ser^5..... Ser^{17}	NC59
b	Ala^1, Thr^3, Tyr^4, Arg^5..... Ser^{17}	ND28
c	IIe^1, thr^3, Arg^4, Ser^5..... Ser^{17}	NC95
d	Arg^4, ... Ser^{17}	Arg4S

* Amino acids after substitution. The superscripts indicate the position numbers of the relevant amino acids from the N terminus.

Following dissolution of the starting material in an aqueous solution such as Tris hydrochloride buffer or phosphate buffer and addition of an enzyme, the enzymatic reaction is carried out at 10-37°C for 30 minutes to 3 days.

The total protein quantity and the protein quantity are determined by the following methods:

The total protein determination is performed by the method of M. M. Bradford [M. M. Bradfor: Anal. Biochem., 72, 248 (1976)].

The protein quantity is determined by SDS-polyacrylamide gel electrophoresis by the method of Laemmli [U. K. Laemmli: Nature, 227, 680 (1970)] followed by measurement on a chromatoscanner (Shimadzu CS-930).

The N-terminal amino acid sequence of the peptide obtained after enzymatic cleavage is determined using an automatic amino acid sequencer "Gas-Phase Protein Sequencer Model 470A" (Applied Biosystems) in combination with a Spectra Physics high-performance liquid chromatograph.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following examples are illustrative of the invention.

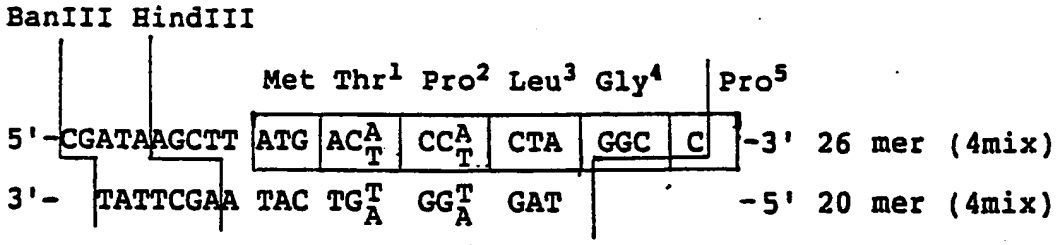
Example 1

Construction of the hG-CSF expression plasmid pCfTA1 (cf. Fig. 1)

A 2-µg portion of the pCSF1-2 DNA obtained in Refernce Example 1 was dissolved in a total amount of 20 µl of a solution (hereinafter referred to as "Y-100 buffer") containing 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 6 mM 2-mercaptoethanol and 100 mM NaCl, 10 units each of the restriction enzymes ApaI (Boehringer Mannheim) and BamHI (Takara Shuzo; hereinafter, unless otherwise specified, all the restriction enzymes used were obtained from Takara Shuzo) were added, and the reaction was carried out at 37°C for 4 hours. From the reaction mixture, there was purified and recovered 0.4 µg of a 1.5 kb DNA fragment by the LGT method.

Separately, 2 µg of the plasmid pLSA1 prepared by the method of Reference Example 3 was dissolved in 20 µl of Y-100 buffer, 10 units each of the restriction enzymes BanIII (Toyobo) and BamHI were added, and the reaction was carried out at 37°C for 4 hours. From this reaction mixture, there was purified and recovered 0.8 µg of a 2.8 kb DNA fragment by the LGT method.

On the other hand, the following DNA linker was synthesized to provide the codons coding for the first to fifth N-terminal amino acids of the mature hG-CSF polypeptide [threonine[1] (ACA or ACT), proline[2] (CCA or CCT), leucine[3] (CTA), glycine[4] (GGC) and proline[5] (CCC)] and the initiation codon (ATG) required for the expression and for adjusting the distance between the SD sequence and ATG downstream from the tryptophan promoter (Ptrp) to an appropriate length between 6-18 bp:



First, the 26-mer and 20-mer single-strand DNAs were synthesized by the phosphotriester method [R. Crea et al.: Proc. Nat. Acad. Sci. USA, 75, 5765 (1978)]. The 26-mer and 20-mer (each 2 µg) were dissolved in 40 µl of a buffer (hereinafter referred to as "T4 kinase buffer") containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM EDTA and 1 mM ATP, 30 units of T4 polynucleotide kinase (Takara Shuzo; hereinafter the same shall apply) was added, and the phosphorylation reaction was carried out at 37°C for 60 minutes.

In 25 µl of a buffer (hereinafter referred to as "T4 ligase buffer") containing 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM dithiothreitol and 1 mM ATP, there were dissolved 0.4 µg of the pCSF1-2-derived ApaI-BamHI fragment (1.5 kb) obtained in the above manner and 0.2 µg of the pLSA1-derived BanIII-BamHI fragment (2.8 kb) obtained in the above manner; 0.1 µg of the above-mentioned DNA linker was added to the mixture. To this mixed solution, there was further added 6 units of T4 DNA ligase (obtained from Takara Shuzo; hereinafter the same shall apply), and the ligation reaction was carried out at 4°C for 18 hours.

The thus-obtained recombinant plasmid mixture was used to transform E. coli HB101 [Bolivar et al.: Gene, 2, 75 (1977)] by the method of Cohen at al. [S. N. Cohen et al.: Proc. Natl. Acad. Aci. USA, 69, 2110 (1972)] (hereinafter, this method was used for transforming E. coli), and an Ap[r] colony was obtained. The plasmid DNA was recovered from the cultured cells of this colony by the known method [H. C. Birnboim et al.: Nucleic Acids Res., 7, 1513 (1979)] (hereinafter, this method was used for plasmid DNA separation). The structure of the plasmid obtained was confirmed by cleavage with Ban III, RsaI, PstI HindIII and BglII followed by agarose gel electrophoresis. This plasmid is called pCfTA1. The base sequence of pCfTA1 in the neighborhood of the BanIII and HindIII sites was confirmed to be as follows by the dideoxy sequencing method using M13 phage:

Example 2

Construction of the plasmid pCfTB20 lacking in part of the 3'-nontranslational region of the hG-CSF cDNA

In 20 µl of Y-100 buffer, there was dissolved 2 µg of the hG-CSF expression plasmid pCfTA1 (4.3 kb) obtained in Example 1, 4 units of the restriction enzyme BamHI was added, and the digestion reaction was carried out at 37°C for 4 hours. After extraction with a mixture of an equal volume of phenol and chloroform (hereinafter referred to as phenol-chloroform extraction), 1.8 µg of a DNA fragment was recovered by precipitation with ethanol. This DNA fragment was dissolved in 20 µl of a buffer (hereinafter referred to as "Klenow buffer") containing 50 mM Tris-HCl (pH 7.8), 7 mM $MgCl_2$ and 6 mM mercaptoethanol, then dATP, dTTP, dCTP and dGTP were added each to a concentration of 1 mM and, after further addition of 4 units of DNA polymerase I Klenow fragment (obtained from Takara Shuzo; hereinafter the same shall apply), and the reaction was carried out at room tmeperature for 1 hour to thereby convert the protruding ends to blunt ends. After phenol-chloroform extraction, 1.6 µg of a DNA fragment was recovered by ethanol precipitation. This DNA fragment was dissolved in 20 µl of Y-100 buffer, 10 units of EcoRI was added, and the cleavage reaction was carried out at 37°C for 4 hours. From the reaction mixture, there was obtained 1 µg of a 2.5 kb DNA fragment [BamHI(blunt)-EcoRI fragment] by the LGT method.

Separately; 2 µg of pCfTA1 was dissolved in 20 µl of Y-100 buffer, 10 units of EcoRI was added, and the cleavage reaction was carried out at 37°C for 4 hours. Thereafter, NaCl was added to an NaCl concentration of 150 mM, then 10 units of DraI was added, and the cleavage reaction was carried out at 37°C for 4 hours. After confirmation of complete cleavage by agarose gel electrophoresis, 0.2 µg of an hG-CSF cDNA-containing 1.0 kb DNA fragment (EcoRI-DraI fragment) was purified and recovered by the LGT method.

In 25 µl of T4 ligase buffer, there were dissolved 0.2 µg of the BamHI (blunt)-EcoRI fragment (2.5 kb) and 0.2 µg of the EcoRI-DraI fragment (1.0 kb) each obtained in the above manner, 6 units of T4 DNA ligase was added to the resultant mixture, and the ligation reaction was carreid out at 4°C for 18 hours.

The thus-obtained recombinant plasmid mixture was used to transform E. coli HB101, and an Ap^r colony was obtained. From cultured cells derived from this colony, a plasmid DNA was recovered. The structure of the plasmid obtained was confirmed by agarose gel electrophoresis following cleavage with HindIII and Pst I. This plasmid is called pCfTB20.

Example 3

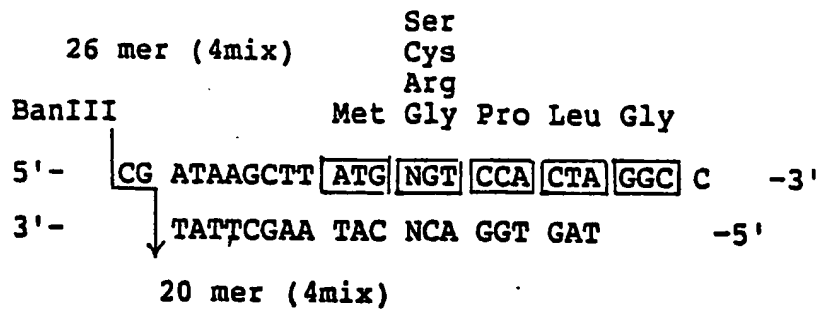
Construction of the plasmids coding for polypeptides resulting from substitution of the N-terminal amino acid of hG-CSF, namely pCfTL23, pCfTL38, pCfTL35 and pCfTL41 (cf. Fig. 3)

In 60 µl of Y-100 buffer, there was dissolved 3 ug of pCSF1-2 (4.5 kb) obtained by the method of Reference Example 1, 8 units each of the restriction enzymes ApaI (Boehringer Mannheim) and Bam HI were added, and the cleavage reaction was carried out at 37°C for 3 hours. From this reaction mixture, there was obtained about 0.4 µg of a DNA fragment of about 1.5 kb (ApaI-BamI fragment) containing most of the hG-CSF gene.

Separately, 2 µg of pGEL1 [Sekine et al.: Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)] (obtained from a culture of E. coli IGEL1 FERM BP-629 by the conventional method) (3.4 kb) was dissolved in 40 µl of Y-100 buffer, 4 units each of the restriction enzymes HindIII, BamHI and PstI were added, and the cleavage reaction was carried out at 37°C for 3 hours. From the reaction mixture, there was obtained about 0.5 µg of a DNA fragment of about 1.7 kb (PstI-BamHI fragment) containing the lipoprotein-derived terminator by the LGT method.

Separately, 3 µg of pKYP10 prepared by the method described in Japanese Patent Application (OPI) NO. 110600/83 was dissolved in 60 µl of Y-100 buffer, 6 units each of the restriction enzymes BanIII (Toyobo) and PstI were added, and the cleavage reaction was carrried out at 37°C for 3 hours. From the reaction mixture, there was obtained about 0.5 ug of a DNA fragment of about 1.1 kb (BanIII-PstI fragment) containing the tryptophan promoter (Ptrp) by the LGT method.

On the other hand, in view of the necessity of substituting the N-terminal amino acid of mature hG-CSF, namely Thr, with Ser, Cys, Arg or Gly and providing the initiation codon (ATG) required for expression and also in view of adjusting the distance between the SD sequence and ATG downstream from Ptrp to an appropriate length of 6-18 bp, and for other reasons, the following DNA linker was synthesized:



In the above formula, N is one of the bases G, A, T and C.

First, the 26-mer and 20-mer single-strand DNAs were synthesized by the ordinary phosphotriester method. The 26-mer and 20-mer (each 20 picomoles) were dissolved in 40 µl of T4 kinase buffer, 6 units of T4 polyuncleotide kinase (Takara Shuzo) was added, and the phosphorylation reaction was carried out at 37°C for 60 minutes.

Then, 0.3 µg of the pCSF1-2-derived ApaI-BamHI fragment (about 1.5 kb), 0.2 µg of the pGEL1-derived PstI-BamHI fragment (about 1.7 kb) and 0.2 µg of the expression vector pKY10-derived BanIII-PstI fragment (about 1.1 kb), each obtained in the above manner, were dissolved in a total of 30 µl of T4 ligase buffer, and about 1 picomole of the above DNA linker was added to the mixture solution. After further addition of 6 units of T4 DNA ligase to the solution, the ligation reaction was carried out at 4°C for 18 hours.

The recombinant plasmid-containing reaction mixture was used to transform E. coli C600SF8 (FERM BP 1070) [Cameron et al.: Proc. Natl. Acad. Sci. USA, 72, 3416 (1975)], and Ap^r colonies were obtained. From these transformants, there were separated and purified the plasmid DNAs by known methods. The structure of each of the plasmid DNAs was confirmed by cleavage with PstI, EcoRI and BanIII, followed by polyacrylamide gel electrophoresis. The plasmids obtained in this way are called pCfTL23, pCfTL38, pCfTL35 and pCfTL41, as shown in Fig. 3. The sequences in the vicinity of the N terminus of the hG-CSF derivative genes in said plasmids were confirmed by the dideoxy sequencing method using M13 phage to be as follows:

	Met Gly Pro Leu Gly Pro Ala
pCfTL23	ATG GGT CCA CTA GGC CCT GCC
	Met Ser Pro Leu Gly Pro Ala
pCfTL38	ATG AGT CCA CTA GGC CCT GCC
	Met Cys Pro Leu Gly Pro Ala
pCfTL35	ATG TGT CCA CTA GGC CCT GCC
	Met Arg Pro Leu Gly Pro Ala
pCfTL41	ATG CGT CCA CTA GGC CCT GCC

The substitution of the N-terminal Thr of mature hG-CSF was confirmed in the pCfTL23-encoded hG-CSF derivative, which is called hG-CSF[Gly^1]. Similarly, N-terminal amino acid substitution by Ser was confirmed in the pCfTL38-encoded hG-CSF derivative, which is called hG-CSF[Ser^1], substitution by Cys in the pCfTL35 encoded hG-CSF derivative, which is called hG-CSF[Cys^1], and substitution by Arg in the pCfTL41 encoded hG-CSF derivative, which is called hG-CSF[Arg^1].

Example 4

Construction of plasmids, DCfTM14, pCfTM17 and pCfTM113, which code for polypeptides resulting from substitution of the N-terminal and third amino acids of hG-CSF (cf. Fig. 4)

In 60 µl of Y-100 buffer, there was dissolved 3 µg of pCSF1-2 (4.5 kb) obtained by the procedure of Reference Example 1, 8 units each of ApaI and BamHI were added, and the cleavage reaction was carried out at 37°C for 3 hours. From this reaction mixture, there was obtained about 0.4 µg of a DNA fragment of about 1.5 kb (ApaI-BamHI fragment) containing most of the hG-CSF gene by the LGT method.

Separately, 2 µg of pGEL1 (3.4 kb) was dissolved in 40 µl of Y-100 buffer, 4 units each of the restriction enzymes HindIII, BamHI and PstI were added, and the cleavage reaction was carried out at 37°C for 3 hours. From this reaction mixture, there was obtained about 0.5 µg of a DNA fragment of about 1.7 kb (PstI-BamHI fragment) containing the lipoprotein terminator by the LGT method.

Further, separately, 3 µg of pKYP10 prepared by the procedure described in Japanese Patent Application (OPI) No. 110600/83 was dissolved in 60 µl of Y-100 buffer, 6 units each of the restriction enzymes BanIII and PstI were added, and the cleavage reaction was conducted at 37°C for 3 hours. From this reaction mixture, there was obtained, by the LGT method, about 0.5 µg of a Ptrp-containing DNA fragment of about 1.1 kb (BanIII-Pst I fragment).

In view of the necessity of substituting the N-terminal amino acid Thr of mature hG-CSF with Ser and the third amino acid Leu of Mature hG-CSF with one of Gly, Ser, Cys and Arg and providing the initiation codon (ATG) required for expression and also in view of the necessity of adjusting the distance between the SD sequence and ATG downstream from Ptrp and appropriate length of 6-18 bp and for other reasons, the following DNA linker was synthesized:

In the above formula, N is one of the bases G, A, T and C.

```
                    (4mix)                          Gly
                    26-mer                          Ser
                                                    Cys
                              Met  Ser  Pro  Arg  Gly
5' - CG ATA AGCTT            [ATG][AGT][CCA][NGT][GGC] C - 3'
   3' - TAT TCGAA             TAC  TCA  GGT  NCA  - 5'
        20 - mer (4mix)
```

First, the 26-mer and 20-mer single-strand DNAs were synthesized by the ordinary phosphotriester method. The 26-mer and 20-mer (each 20 picomoles) were dissolved in 40 µl of T4 kinase buffer, 6 units of T4 polynucleotide kinase was added, and the phosphorylation reaction was carried out at 37°C for 60 minutes.

Then, 0.3 µg of the pCSF1-2-derived ApaI-BamHI fragment (about 1.5 kb), 0.2 µg of the pGEL1-derived PstI-BamHI fragment (about 1.7 kb) and 0.2 µg of the BanIII-Pst I fragment (about 1.1 kb) of the expression vector pKYP10, each obtained in the above manner, were dissolved in 30 µl of T4 ligase buffer, and about 1 picomole of the above DNA linker was added to the mixture solution. After further addition of 6 units of T4 DNA ligase to the solution, the ligation reaction was carried out at 4°C for 18 hours.

The recombinant plasmid-containing reaction mixture was used to transform E. coli C600SF8 (FERM BP-1070) by the method of Cohen et al. and Ap^r colonies were obtained. The plasmid DNAs were separated and purified from these transformants by known methods. The structure of each of said plasmid DNAs was confirmed by cleavage with PstI, EcoRI and BanIII, followed by polyacrylamide gel electrophoresis. The plasmids obtained in the above manner are called pCfTM14, pCfTM17 and pCfTM113, as shown in Fig. 4. The sequences in the vicinity of the N terminus of the hG-CSF derivative-encoding genes were confirmed by the dideoxy sequencing method using M13 phage to be as follows:

		Met	Ser	Pro	Cys	Gly	Pro	Ala
pCfTM14	-	ATG	AGT	CCA	TGT	GGC	CCT	GCC
		Met	Ser	Pro	Arg	Gly	Pro	Ala
pCfTM17	-	ATG	AGT	CCA	CGT	GGC	CCT	GCC
		Met	Ser	Pro	Ser	Gly	Pro	Ala
pCfTM113	-	ATG	AGT	CCA	AGT	GGC	CCT	GCC

The substitution of the N-terminal Thr and third amino acid Leu of mature hG-CSF by Ser and Cys, respectively was confirmed in the pCfTM14-encoded derivative, which is called hG-CSF[Ser^1, Cys^3]. Similarly, the substitution of the N-terminal Thr and third amino acid Leu by Ser and Arg, respectively was confirmed in the pCfTM17-encoded derivative, which is called hG-CSF[Ser^1, Arg^3], and the substitution of the N-terminal Thr and third amino acid Leu by Ser and Ser, respectively in the pCfTM113-encoded derivative, which is called hG-CSF[Ser^1, Ser^3].

Example 5

(1) Construction of the recombinant plasmid pCfWD1 (cf. Fig. 5)

In 50 µl of Y-100 buffer, there was dissolved 5 µg of pCfTA1 obtained by the procedure of Example 1, 10 units of the restriction enzyme Stu I and 10 units of the restriction enzyme BanIII (Toyobo) were added, and the digestion reaction is carried out at 37°C for 1 hour. From the reaction mixture, there was obtained about 0.5 pg of an hG-CSF cDNA-containing DNA fragment of about 1.3 kb (BanIII-Stu I fragment). Separately, 3 µg of pKYP26 produced by the procedure of Reference Example 2 was dissolved in 50 µl of Y-100 buffer, 6 units of BamHI was added, and the digestion reaction was carried out at 30°C for 1 hour.

To this was added an equal volume of phenol saturated with 10 mM Tlrs-HCl (pH 7.5) and 1 mM EDTA. After vigorous stirring, the aqueous layer was collected by low-speed centrifugation (3,300 rpm, 10 minutes; hereinafter, the same conditions were used). An equal volume of chloroform was added and, after vigorous stirring, the aqueous layer was collected by low-speed centrifugation. A 1/10 volume of 3 M sodium acetate was added, 2.5 volumes of ethanol was then added, and the mixture was allowed to stand at -20°C for 1 hour. The precipitate was collected by cold centrifugation (4°C, 11,000 rpm, 10 minutes). This precipitate was dissolved in 30 µl of Klenow buffer, dATP, dTTP, dCTP and dGTP were added each to a concentration of 100 µM, 2 units of DNA polymerase I Klenow fragment was added, and the reaction was carried out at 17°C for 15 minutes. The DNA polymerase I Klenow fragment was inactivated by treating at 68°C for 10 minutes, thereafter NaCl was added to a concentration of 100 mM, 5 units of the restriction enzyme Pst I was added, and the digestion reaction was carried out at 37°C for 1 hour. From the reaction mixture, there was obtained, by the LGT method, about 0.6 µg of an *t*pp terminator-containing DNA fragment of about 1.8 kb [BamHI (blunt)-PstI fragment]. Separately, 4 µg of pGEL1 was dissolved in 40 µl of Y-100 buffer, 10 units each of the restriction enzymes BanIII (Toyobo) and PstI were added, and the digestion reaction was conducted at 37°C for 1 hour, and 0.4 µg of tryptophan promoter-containing DNA fragment of about 1 kb (BanIII-PstI fragment) was obtained from the reaction mixture by the LGT method.

About 0.2 µg of the pCfTA1-derived BanIII-StuI fragment (about 1.3 kb), about 0.1 µg of the pKYP26-derived BamHI(blunt)-PstI fragment (about 1.8 kb) and about 0.1 pg of the pGEL1-derived BanIII-PstI fragment (about 1 kb) were dissolved in 30 µl of T4 DNA ligase buffer, 4 units of T4 DNA ligase was added, and the ligation reaction was performed at 4°C for 18 hours.

The reaction mixture was used to transform *E. coli* HB101 and an Ap^r colony was obtained, and the plasmid DNA was recovered from this colony by the above-mentioned method of Bimboim et al. Thus was obtained pCfWD1 shown in Fig. 5.

(2) Construction of pCfT95K19 (cf. Fig. 6)

In 50 µl of Y-100 buffer, there was dissolved 5 µg of the pCfTL38 obtained by the procedure of Example 3, 10 units each of the restriction enzymes HindIII and BglIII were added, and the digestion reaction was carried out at 37°C for 1 hour. About 0.7 pg of a tryptophan promoter-containing DNA fragment of about 2.6 kb (HindIII-BalII fragment) was obtained from the reaction mixture by the LGT method. Separately, 100 µg of pCfTL38 was dissolved in 1.5 ml of Y-100 buffer, 80 units each of the restriction enzymes BamHI and HindIII were added, and the digestion reaction was conducted at 37°C for 6 hours. An hG-CSF cDNA-containing DNA fragment was recovered from the reaction mixture by the LGT method and purified using ELUTIPTM-d (Schleicher & Schuell). This DNA fragment was dissolved in a totoal volume of 90 µl of a solution containing 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 150 mM NaCl and 6 mM 2-mercaptoethanol (hereinafter referred to as "Y-150 buffer"), 3 units of the restriction enzyme DpnI (Boehringer Mannheim) was added, and the digestion reaction was carried out at 37°C for 15 minutes. About 1 µg of an hG-CSF cDNA-containing DNA fragment of about 300 bp (HindIII-DpnI fragment) was obtained from the reaction mixture by polyacrylamide gel electrophoresis.

Separately, 10 µg of pCfTB20 obtained by the procedure of Example 2 was dissolved in 100 µl of Y-100 buffer, 10 units of the restriction enzyme AvaI was added, and the digestion reaction was performed at 37°C for 1 hour. The DNA recovered from the digest by phenol-chloroform extraction and ethanol precipitation was dissolved in 30 µl of Klenow buffer, 2 units of DNA polymerase I Klenow fragment was added, and the reaction was carried out at 17°C for 30 minutes. The DNA polymerase I Klenow fragment was inactivated by treating at 68°C for 10 minutes, NaCl was added to 100 mM, 10 units of the restriction enzyme BglII was added, an the digestion reaction was conducted at 37°C for 1 hour. About 0.3.µg of an trp terminator portion-containing DNA fragment of about 480 bp [AvaI(blunt)-BglII fragment] was obtained from the reaction mixture by the LGT method.

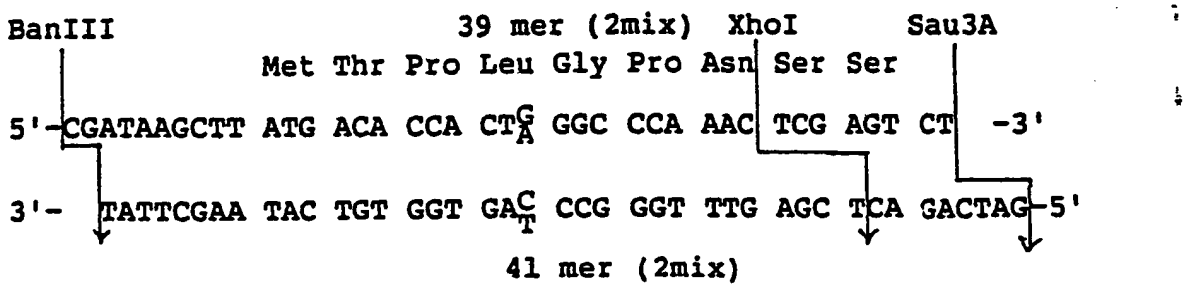
In 30 µl of T4 DNA ligase buffer, there were dissolved about 0.1 µg of the pCfTL38-derived HindIII-BglII fragment (about 2.6 kb), about 0.2 µg of the pCfTL38-derived HindIII-DpnI fragment (about 300 bp) and about 0.15 µg of the pCfTB20-derived AvaI(blunt)-BglII fragment (about 480 bp), each obtained in the above manner, and, after addition of 4 units of T4 DNA ligase, the ligation reaction was carried out at 4°C for 18 hours. The reaction mixture was used to transform E. coli HB101 and an Ap^r colony was obtained. From this colony, there was recovered the plasmid DNA by the above mentioned method of Birnboim et al. Thus was obtained pCfT95K19 shown in Fig. 6.

(3) Construction of pCfAA1 (of. Fig. 6)

In 50 µl of Y-100 buffer was dissolved 5 µg of pCfT95K19 obtained as described in the preceding section. Thereto were added 7 units of the restriction enzyme BanIII (Toyobo) and 2 units of BglI (Nippon Gene), and the digestion reaction was conducted at 37°C for 1 hour. From the reaction mixture, there were obtained, by the LGT method, about 0.6 µg of tryptophan promotoer portion-containing DNA fragment of about 1 kb (BanIII-BglI fragment) and about 1 µg of an trp terminator portion-containing DNA fragment of about 1.8 kb (BglI-BglI fragment).

Separately, 15 µg of pCfT95K19 was dissolved in 150 µl of Y-100 buffer, 6 units of the restriction enzyme BglI (Nippon Gene) and 10 units of Sau3A were added, and the digestion reaction was carried out at 37°C for 1 hour. Polyacrylamide gel electrophoresis of the reaction mixture gave about 0.3 µg of an hG-CSF cDNA portion-containing DNA fragment of about 350 bp (BgI I-Sau3A fragment).

Further, separately, the following DNA linker was synthesized:



First, the 39-mer and 41-mer single-strand DNAs were synthesized by the ordinary phosphotriester method. The 39-mer and 41-mer (each 20 picomoles) were dissolved in a total volume of 40 µl of T4 DNA kinase buffer, 6 units of T4 polynucleotide kinase (Takara Shuzo) was added, and the phosphorylation reaction was carried out at 37°C for 60 minutes.

Then, 0.1 µg of the pCfT95K19-derived Ban III-BglI fragment (about 1 kb), 0.05 µg of the BglI-BglI fragment (about 1.8 kb) and 0.1 µg of the BglI-Sau3A fragment (about 350 bp), each obtained in the above manner, were dissolved in 25 µl of T4 DNA ligase buffer, followed by addition of about 2 picomoles of the above DNA linker. After further addition of 6 units of T4 DNA ligase, the ligation reaction was conducted at 4°C for 18 hours.

The reaction mixture was used to transform E. coli HB101, an Ap^r colony was obtained, and the plasmid DNA was recovered from this colony by the above-mentioned method of Birnboim et al. Thus was obtained pCfAA1 shown in Fig. 6. Determination of the base sequence of the linker portion of pCfAA1 by the above-mentioned dideoxy sequencing method revealed that the third base of the codon coding for the fourth amino acid Leu is A. In this pCfAA1, the DNA portion coding for the 14 amino acids from the 10th amino acid Pro to the 23rd amino acid Lys of hG-CSF is missing. Furthermore, such mutation has been introduced as to change the 6th amino acid of hG-CSF from A1a to Asn, and there is now a new XhoI site.

(4) Construction of pCfAB5 (cf. Fig. 6

In 30 µl of Y-100 buffer was dissolved 3 µg of pCfAA1 obtained as described in the previous section, 5 units of the restriction enzyme XhoI was added, and the digestion reaction was carried out at 37°C for 1 hour. After confirmation of complete XhoI cleavage by agarose gel electrophoresis, 1 unit of the restriction enzyme BglI (Nippon Gene) was added, and partial digestion was effected at 37°C for 25 minutes. From the reaction mixture, there was obtained, by the LGT method, about 1µg of a tryptophan promoter portion- and tpp terminator portion-containing DNA fragment of about 3 kb (XhoI-BglI fragment). Separately, the following DNA linker was synthesized:

```
XhoI
   Ser Ser Leu Pro Gln Ser Phe Leu
   TCG AGT CTA CCA CAG AGC TTC CT          23 mer
      CA GAT GGT GTC TCG AAG GAAAATTT      25 mer

          (Cys
           Ser
           Gly)                  Sau3A
  Leu Lys Arg Leu Glu Gln Val Arg Lys
 TTTA AAA NGC TTA GAG CAA GTG AGG  AA      27 mer (4mix)
       T NCG AAT CTC GTT CAC TCC  TTCTAG    25 mer (4mix)
```

(N being G, A, T or C).

This linker DNA contains that DNA portion which codes for the 14 amino acids of hG-CSF from the 10th amino acid Pro to the 23rd amino acid Lys. Such portion is missing in the hG-CSF cDNA of pCfAA1.

First, the 27-mer, 25-mer (two kinds) and 23-mer single-strand DNAs were synthesized by the ordinary phosphotriester method. The 27-mer and 25-mer DNAs complementary to each other and the 25-mer and 23-mer DNAs complementary to each other were dissolved in pairs, and each in an amount of 20 picomoles, in a total volume of 40 µl of T4 kinase buffer; 6 units of T4 polynucleotide kinase (Takara Shuzo) was added to each solution, and the phosphorylation reaction was performed at 37°C for 60 minutes.

Then, 0.1 µg of the pCfAA1-derived XhoI-BglI fragment (about 3 kb) obtained as described above and 0.1 µg of the pCfT95K19-derived BglI-Sau3A fragment (about 350 bp) obtained as described in the previous section were dissolved in 30 µl of T4 DNA ligase buffer, and 2 picomoles each of the above DNA linker portions were added to the mixture solution. Further, 6 units of T4 DNA ligase was added, and the ligation reaction was conducted at 4°C for 18 hours.

The reaction mixture was used to transform E. coli HB101 and Ap^r colonies were obtained. From these

colonies, the plasmid DNAs were recovered by the above-mentioned method of Birnboim et al. There were thus obtained pCfAB5 and pCfAB14 shown in Fig. 6. Determination of the base sequence of the DNA linker moiety of pCfAB5 and of pCfAB14 by the above-mentioned dideoxy sequencing method revealed that the first base of the codon coding for the 17th amino acid is A in pCfAB5 and T in pCfAB14, hence said codon in for Ser (AGC) in the former and for Cys (TGC) in the latter, leading to substitution of Ser for the 17th amino acid Cys of mature hG-CSF in pCFAB5, but no substitution in pCfAB14.

Example 6

(1) Construction of pCfBA8 and pCfBA32 (cf. Fig. 7)

In 40 µl of Y-100 buffer waqs dissolved 3 µg of pCfAB5 obtained as described in the previous section, 5 units each of the restriction enzymes AvaI and BglII were added, and the digestion reaction was conducted at 37°C for 1 hour. From the reaction mixture, there was obtained, by the LGT method, about 1 µg of a tryptophan promoter portion-and ℓpp terminator portion-containing DNA fragment of about 2.8 kb (AvaI-BglII fragment).

Separately, 6 µg of pCfWD1 obtained as described in section 1 was dissolved in 50 µl of Y-100 buffer, 5 units of the restriction enzyme BglII was added, and the digestion reaction was carried out at 37°C for 1 hour. Agarose gel electrophoresis confirmed that the cleavage with BglII was complete. Thereafter, 3 units of the restriction enzyme AvaI was added, and partial cleavage was effected at 37°C for 20 minutes. From the reaction mixture, there was obtained, by the LGT method, 0.4 µg of a DNA fragment (about 1.3 kb) containing most of the hg-CSF (Bgl II-AvaI fragmnent).

Then, 0.1 µg of the pCfAB5-derived AvaI-BglII fragment (about 2.8 kb) and 0.3 µg of the pCfWD1-derived BglII-AvaI fragment (about 1.3 kb), each obtained as described above, were dissolved in 25 µl of T4 DNA ligase was added, and the ligation reaction was carried out at 4°C for 18 hours.

The reaction mixture was used to transform E. coli HB101 and an Ap^r colony was obtained. From this colony, the plasmid DNA was recovered by the above-mentioned method of Bimboim et al. Thus was obtained pCfBA8.

The amino acid sequence of the hG-CSF derivative encoded by pCfBA8 contains Asn in place of the 6th amino acid A1a of mature hG-CSF and Ser in place of the 17th amino acid Cys thereof. Hereinafter, this derivative is referred to as hG-CSF[NA8].

On the other hand, 3 µg of pCfAB14 obtained as described in the previous section was dissolved in 40 µl of Y-100 buffer, 5 units each of the restriction enzyme AvaI and BglII were added, and the digestion reaction was performed at 37°C for 1 hour. From the reaction mixture, there was obtained, by the LGT method, about 1 µg of a tryptophan promoter portion-and ℓpp terminator portion-containing DNA fragment of about 2.8 kb (AvaI-BglII fragment).

Separately, 6 µg of pCfWD1 obtained as described in section 1 was dissolved in 50 µl of Y-100 buffer, 5 units of the restrictrion enzyme BglII was added, and the digestion reaction was carried out at 37°C of 1 hour. After confirmation of the completeness of the BglII cleavage by agarose gel electrophoresis, 3 units of the restriction enzyme AvaI was added, and partial cleavage was effected at 37°C for 20 minutes. From the reaction mixture, there was obtained, by the LGT method, 0.4 µg of a DNA fragment (about 1.3 kb) containing most of the hG-CSF cDNA (BglII-AvaI fragment).

Then, 0.1 µg of the pCfAB14-derived AvaI-Bgl II fragment (about 2.8 kb) and 0.3 µg of the pCfWD1-derived Bgl II-AvaI fragment (about 1.3 kb), each obtained as described above, were dissolved in 25 µl of T4 DNA ligase buffer, 3 units of T4 DNA ligase was added, and the ligation reaction was performed at 4°C for 18 hours.

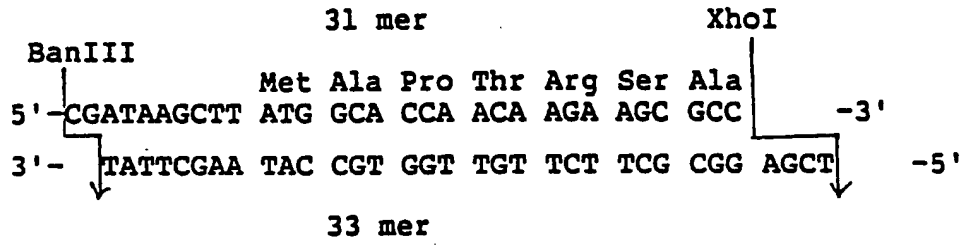
The reaction Mixture was used to transform E. coli HB101 and an Ap^r colony was obtained and, from this colony, the plasmid DNA was recovered by the above-mentioned method of Birnboim et al. Thus was obtained pCfBA32 shown. in Fig. 7.

The amino acid sequence of the hG-CSF derivative encoded by pCfBA32 contains Asn in lieu of the 6th amino acid A1a of mature hG-CSF.

(2) Construction of pCfBB101

In 50 µl of Y-100 buffer was dissolved 6 µg of pCfBA8 obtained as described in the previous section, 10 units of the restriction enzyme BanIII (Toyobo), 8 units of BglII and 8 units of XhoI were added, and the digestion reaction was conducted at 37°C for 1 hour. From the reaction mixture, there were obtained, by the LGT method, about 0.6 µg of an hG-CSF cDNA-containing DNA fragment of about 1.4 kb (XhoI-BglII fragment) and about 0.8 µg of a tryptophan promoter portion-containing DNA fragment of about 2.7 kb (BanIII-BglII fragment).

Separately, the following DNA linker was synthesized:



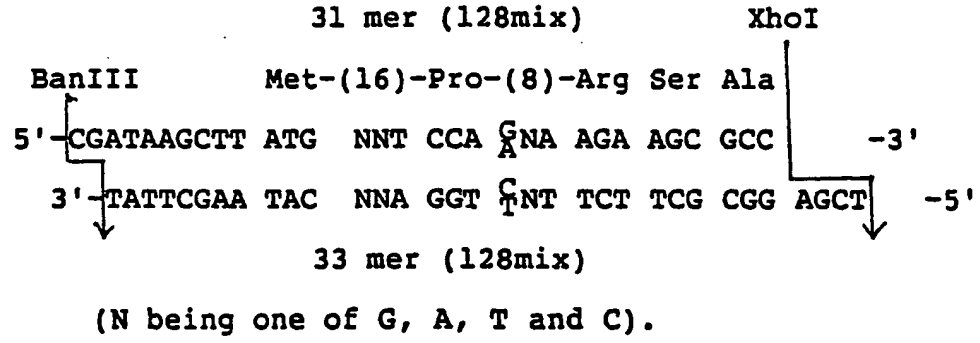
First, the 31-mer and 33-mer single-strand DNAs were synthesized by the ordinary phosphotriester method. The 31-mer and 33-mer (each 2 µg) were dissolved in a total of 40 µl of T4 kinase buffer, 30 units of T4 polynucleotide kinase (Takara Shuzo) was added, and the phosphorylation reaction was conducted at 37°C for 60 minutes.

Then, 0.1 µg of the pCfBA8-derived BanIII-BglII fragment (about 2.7 kb fragment) and 0.1 µg of the pCfBA8-derived XhoI-BglII fragment (about 1.4 kb fragment), each obtained as described above, were dissolved in 25 µl of T4 DNA ligase buffer, and about 2 picomoles of the above DNA linker was added to the mixture solution. After further addition of 6 units of T4 DNA ligase, the ligation reaction was carried out at 4°C for 18 hours.

The recombinant plasmid mixture thus obtained was used to transform E. coli HB101 and an Ap^r colony was obtained. From cultured cells derived from this colony, there was recovered the plasmid DNA. Thus was obtained pCfBB101 shown in Fig. 7. The amino acid sequence of the hG-CSF derivative encoded by pCfBB101 contains Ala, Thr, Arg, Ser and Ser in lieu of the first amino acid Thr, third amino acid Leu, fourth amino acid Gly, fifth amino acid Pro and 17th amino acid Cys of mature hG-CSF, respectively. Hereinafter, this derivative is referred to as hG-CSF[NB101].

(3) Construction of DCfBC42B1, pCfBC45, pCfBC52, pCfBC59, pCfBC76, pCfBC77, pCfBC93, pCfBC95 and pCfBC97 (Cf. Fig. 8)

First, the following DNA linker was synthesized:



In this synthetic DNA linker, the three bases each represented by N are each independently one of G, A, T and C and one base is G or A (in the case of 31-mer) or C or T (in the case of 33-mer) and therefore this linker is obtained as a mixture of a total of 128 DNA linkers. As a result, the design of this linker is such

that, in the N-terminal hG-CSF amino acid sequence encoded by this linker, 16 different amino acids are possible as the amino acid next to Met and 8 different amino acids are possible as the amino acid next to Pro, hence 128 amino acid sequences in total are possible.

First, the 31-mer and 33-mer single-strand DNAs were synthesized by the ordinary phosphotriester method. The 31-mer and 33-mer (each 2 μg) were dissolved in a total volume of 40 μl of T4 kinase buffer, 30 units of T4 polynucleotide kinase (Takara Shuzo) was added, and the phosphorylation reaction was conducted at 37°C for 60 minutes.

Then, 0.1 μg of the pCfBA8-derived BanIII-BglII fragment (about 2.7 kb fragment) and 0.1 μg of the pCfBA8-derived XhoI-BglII frament (about 1.4 kb fragment), each obtained in Example 6, were dissolved in 25 μl of T4 DNA ligase buffer, and about 2 picomoles of the above DNA linker was added to the mixture solution. After further addition of 6 units of T4 DNA ligase, the ligation reaction was carried out at 4°C for 18 hours.

The recombinant plasmid mixture thus obtained was used to transform E. coli HB101 and Ap^r colonies were obtained. From cultured cells of these colonies, the plasmid DNAs were recovered. Thus were obtained pCfBC42B1, pCfBC45, pCfBC52, pCfBC59, pCfBC76, pCfBC77, pCfBC93, pCfBC95 and pCfBC97. Determination of the base sequence in each DNA linker moiety by the above-mentioned dideoxy sequencing method revealed that the base sequences on the N-terminal side of hG-CSF derivatives are as follows:

	Met Thr Pro Glu Lys Ser Ala
pCfBC42B1	ATG ACT CCA GAA AAA AGC GCC
	Met Val Pro Ile Arg Ser Ala
pCfBC45	ATG GTT CCA ATA AGA AGC GCC
	Met Cys Pro Ile Arg Ser Ala
pCfBC52	ATG TGT CCA ATA AGA AGC GCC
	Met Tyr Pro Ile Arg Ser Ala
pCfBC59	ATG TAT CCA ATA AGA AGC GCC
	Met Arg Pro Thr Arg Ser Ala
pCfBC76	ATG CGT CCA ACA AGA AGC GCC
	Met Thr Pro Thr Arg Ser Ala
pCfBC77	ATG ACT CCA ACA AGA AGC GCC
	Met Asn Pro Glu Arg Ser Ala
pCfBC93	ATG AAT CCA GAA AGA AGC GCC
	Met Ile Pro Thr Arg Ser Ala
pCfBC95	ATG ATT CCA ACA AGA AGC GCC
	Met Ser Pro Thr Arg Ser Ala
pCfBC97	ATG AGT CCA ACA AGA AGC GCC

The substituent aqmino acid residues in the hG-CSF derivatives encoded by these plasmids as respectively compared with mature hG-CSF are as follows:

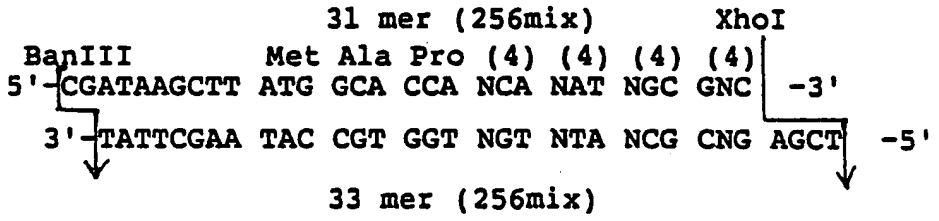
Plasmid	Position of amino acid substitution (amino acid of hG-CSF) 1st (Thr)	3rd (Leu)	4th (Gly)	5th (Pro)	17th (Cys)
pCfBC42B1	-*	Glu	Lys	Ser	Ser
pCfBC45	Val	Ile	Arg	Ser	Ser
pCfBC52	Cys	Ile	Arg	Ser	Ser
pCfBC59	Tyr	Ile	Arg	Ser	Ser
pCfBC76	Arg	Thr	Arg	Ser	Ser
pCfBC77	-*	Thr	Arg	Ser	Ser
pCfBC93	Asn	Glu	Arg	Ser	Ser
pCfBC95	Ile	Thr	Arg	Ser	Ser
pCfBC97	Ser	Thr	Arg	Ser	Ser

* No substitution

The hG-CSF derivatives encoded by pCfBC42B1, pCfBC45, pCfBC52, pCfBC59, pCfBC76, pCfBC77, pCfBC93, pCfBC95 and pCfBC97 are hereinafter referred to as hG-CSF[NC42B1], hG-CSF[NC45], hG-CSF-[NC52], hG-CSF[NC59], hG-CSF[NC76], hG-CSF[NC77], hG-CSF[NC93], hG-CSF[NC95] and hG-CSF-[NC97], respectively.

(4) Construction of pCfBD28, pCfBDD56 and pCfBD82

First, the following DNA linker was synthesized:



(N being one of G, A, T and C)

In this DNA linker, the four bases represented by N are each independently G, A, T or C and, accordingly, the linker is obtained as a mixture of a total of 256 different DNA linkers. As a result, the design of this DNA linker is such that, in the N-terminal hG-CSF amino acid sequence encoded by the DNA linker, four amino acids are possible in each of the four positions in question, hence totally 256 different amino acid sequences are possible.

First the 31-mer and 33-mer single-strand DNAs were synthesized by the ordinary phosphotriester method. In a total of 40 µl of T4 kinase buffer, there were dissolved 2 µg each of the 31-mer and 33-mer,

30 units of T4 polynucleotide kinase (Takara Shuzo) was added, and phosphorylation reaction was performed at 37°C for 60 minutes.

Then, 0.1 µg of the pCfBA8-derived BanIII-BglII fragment (about 2.7 kb fragment) and 0.1 µg of the pCfBA8-derived Xho I-BglII fragment (about 1.4 kb fragment), each obtained in Example 6, were dissolved in 25 µl of T4 DNA ligase buffer, and about 2 picomoles of the above DNA linker was added to the mixture solution. After further addition of 6 units of T4 DNA ligase, the ligation reaction was conducted at 4°C for 18 hours.

The recombinant plasmid mixture obtained was used to transform E. coli HB101, and Ap^r colonies were obtained. From cultured cells of these colonies, the plasmids were respectively recovered. Thus were obtained pCfBD28, pCfBD56 and pCfBD82. Determination of the base sequence in the DNA linker moiety by the above mentioned dideoxy sequencing method revealed that the base sequences on the N-terminal side of the hG-CSF derivatives are as follows:

	Met	Ala	Pro	Thr	Tyr	Arg	Ala
pCfBD28	ATG	GCA	CCA	ACA	TAT	CGC	GCC
	Met	Ala	Pro	Ser	Asn	Ser	Ala
pCfBD56	ATG	GCA	CCA	TCA	AAT	AGC	GCC
	Met	Ala	Pro	Pro	Asn	Arg	Gly
pCfBD82	ATG	GCA	CCA	CCA	AAT	CGC	GGC

The replacing amino acid residues in the hG-CSF derivatives encoded by these plasmids as compared with mature hG-CSF are as follows;

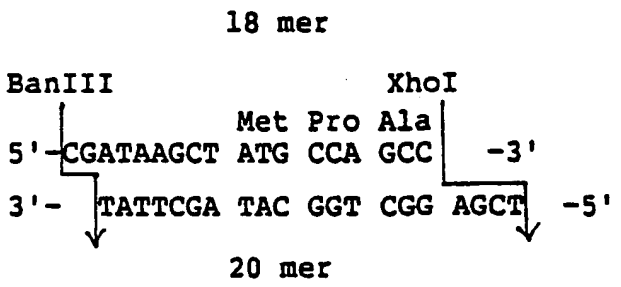
Position of amino acid substitution (amino acid of hG-CSF)	Plasmid pCfBD28	pCfBD56	pCfBD82
1st (Thr)	Ala	Ala	Ala
3rd (Leu)	Thr	Ser	Pro
4th (Gly)	Tyr	Asn	Asn
5th (Pro)	Arg	Ser	Arg
6th (Ala)	-*	-*	Gly
17th (Cys)	Ser	Ser	Ser

* No substitution.

The hG-CSF derivatives encoded by pCfBD28, pCfBD56, and pCfBD82 are hereinafter referred to as hG-CSF[ND28], hG-CSF[ND56] and hG-CSF[ND82], respectively. An E. coli strain harboring pCfBD56, E. coli ECfBD56, and an E. coli strain harboring pCfBD28, E. coli ECfBD28, have been deposited at the Fermentation Research Institute under the deposit numbers FERM BP-1221 and FERM BP-1479, respectively in accordance with the Budapest treaty.

(5) Construction of pCfTNS7 (cf Fig. 14)

The following DNA linker was synthesized.



According to the design of this DNA linker, the four amino acids from the Ist amino acid Thr to the 4th amino acid Gly of the N-terminal amino acid sequence of hG-CSF are missing in the N-terminal amino acid sequence encoded by the linker.

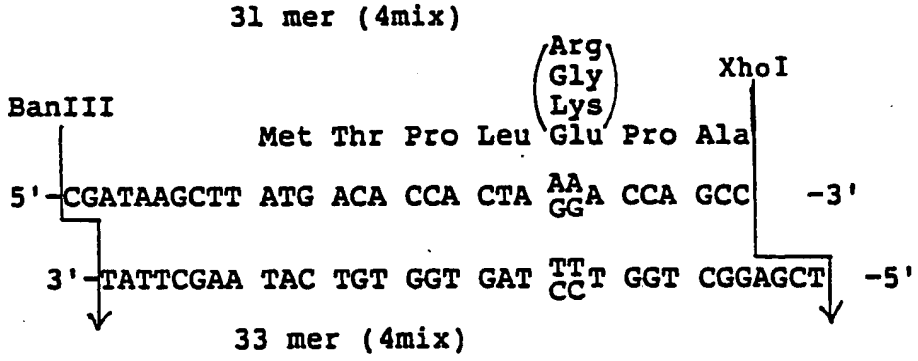
First, the 18-mer and 20 mer single-strand DNAs were synthesized by the ordinary phosphotriester method. The 18 mer and 20-mer (each 2 µg) were dissolved in a total of 40 µl of T4 kinase buffer, 30 units of T4 polynucleotide kinase (Takara Shuzo) was added, and the phosphorylation reaction was performed at 37°C for 60 minutes.

Then, 0.1 µg of the pCfBA8-derived BanIII-BglII fragment (about 2.7 kb fragment) and 0.1 µg of the pCfBA8-derived Xho I-BglII fragment (about 1.4 kb), each obtained in Example 6, were dissolved in 25 µl of T4 DNA ligase buffer, about 2 picomoles of the above DNA linker was added to the mixture solution, and the ligation reaction was conducted at 4°C for 18 hours.

The recombinant plasmid mixture thus obtained was used to transform E. coli HB101, and an Ap^r colony was obtained. From cultured cells of this colony, there was recovered the plasmid. Thus was obtained pCfTNS7. The hG-CSF derivative encoded by pCfTNS7 is hereinafter referred to as hG-CSF[Δ1-4S].

(6) Construction of pCfTAArg4S (cf. Fig. 14)

The following DNA linker was synthesized:



In this DNA linker, two bases each independently is A or G, hence said linker is obtained as a mixture of a total of four DNA linkers. Accordingly, the design of this DNA linker is such that four amino acids are possible as the 4th amino acid in the N-terminal hG-CSF amino acid sequence encoded by said linker.

First, the 31-mer and 33-mer single-strand DNAs were synthesized by the ordinary phosphotriester method. The 31-mer and 33-mer (each 2 µg) were dissolved in a total volume of 40 pl of T4 kinase buffer, 30 units of T4 polynucleotide kinase (Takara Shuzo) was added, and the phosphorylation reaction was carried out at 37°C for 60 minutes.

Then, 0.1 μg of the pCfBA8-derived BanIII-BglII fragment (about 2.7 kb fragment) and 0.1 μg of the pCfBA8-derived XhoI-BglII fragment (about 1.4 kb fragment), each obtained as described in section (1), were dissolved in 25 μl of T4 DNA ligase buffer, and about 2 picomoles of the above DNA linker was added to the mixture solution. After further addition of 6 units of T4 DNA ligase, the ligation reaction was performed at 4°C for 18 hours.

The recombinant plasmid mixture thus obtained was used to transform E. coli HB101, and an Ap^r colony was obtained. From cultured cells of this colony, there was recovered the plasmid. Thus was obtained pCfTAArg4S. Determination of the base sequence of the DNA linker moiety by the above-mentioned dideoxy sequencing method revealed that the N-terminal base sequence of the hG-CSF derivative is as follows:

```
                    Met Thr Pro Leu Arg Pro Ala
pCfTAArg4S  ATG ACA CCA CTA AGA CCA GCC
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The hG-CSF derivative encoded by pCfTAArg4S is hereinafter referred to as hG-CSF[Arg^4,Ser^{17}].

(7) Construction of pCfTN205 (cf. Fig. 15)

In 40 μl of K-150 buffer (same as Y-100 buffer except for replacement of 150 mM KCl for 100 mM NaCl), there was 3 μg of pCfTNS7 obtained in section 5, 5 units each of the restriction enzymes PvuI and XhoI, and the digestion reaction was conducted at 37°C for 1 hour. From the reaction mixture, there was obtained, by the LGT method, about 0.5 μg of a tryptophan promoter portion-containing DNA fragment of about 1.0 kb (PvuI-XhoI fragment).

Separately, 3 μg of pCfBA32 obtained in section 1 was dissolved in 40 μl of K-150 buffer, 5 units each of the restriction enzymes PvuI and XhoI were added, and the digestion reaction was carried out at 37°C for 1 hour. From the reaction mixture, there was obtained, by the LGT method, 2 μg of an about 3.0 kb DNA fragment (XhoI-PvuI fragment) containing most of the hG-CSF cDNA.

Then, 0.1 μg of the pCfTNS7-derived PvuI-XhoI fragment (about 1.0 kb) and 0.3 μg of the pCfBA32-derived XhoI-PvuI fragment (about 3.0 kb), each obtained in the above manner, were dissolved in 25 μl of T4 DNA ligase buffer, 3 units of T4 DNA ligase was added, and the ligation reaction wa conducted at 4°C for 18 hours.

The reaction mixture was used to transform E. coli HB101, and Ap^r colony was obtained, and the plasmid DNA was recovered from this colony by the above-mentioned method of Birnboim et al. Thus was obtained pCfTB205 shown in Fig. 15.

In the amino acid sequence of the hG-CSF derivative encoded by pCfTN205, the 1st to 4th amino acids of mature hG-CSF are missing. Hereinafter, this derivative is referred to as hG-CSF[Δ1-4].

(8) Construction of pCfTAArg4 (cf. Fig. 15)

In 40 μl of K-150 buffer, there was dissolved 3 μg of pCfTAArg4S obtained in section 6, 5 units each of the restriction enzymes PvuI and XhoI were added, and the digestion reaction was performed at 37°C for 1 hour. From the reaction mixture, there was obtained by the LGT method, about 0.5 μg of a tryptophan promoter portion-containing DNA fragment of about 1.0 kb (PvuI-XhoI fragment).

Separately, 3 μg of pCfBA32 obtained in section 1 was dissolved in 40 μl of K-150 buffer, 5 units each of the restriction enzymes PvuI and XhoI were added, and the digestion reaction was conducted at 37°C for 1 hour. From the reaction mixture, there was obtained, by the LGT method, 2 μg of an about 3.0 kb DNA fragment (XhoI-PvuI fragment) containing most of the hG-CSF cDNA.

Then, 0.1 μg of the pCfTAArg4S-derived PvuI-XhoI fragment (about 1.0 kb) and 0.3 μg of the pCfBA32-derived XhoI-PvuI fragment (about 3.0 kb), each obtained in the above manner, were dissolved in 25 μl of T4 DNA ligase buffer, 3 units of T4 DNA ligase was added, and the ligation reaction was carried out at 4°C for 18 hours.

The reaction mixture was used to transform E. coli HB101, Ap^r colony was obtained, and the plasmid

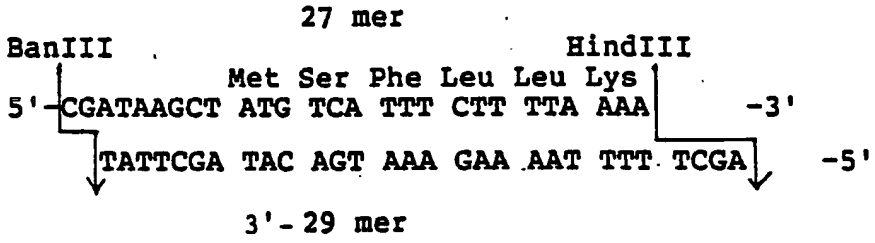
DNA was recovered from this colony by the above-mentioned method of Birnboim et al. Thus was obtained pCfTAArg4 shown in Fig. 15.

The fourth amino acid in the amino acid sequence of the hG-CSF derivative encoded by pCfTAArg4 is Arg in lieu of Gly in mature hG-CSF. Hereinafter, this derivative is called hG-GSF[Arg^4].

(9) Construction of pCfTNS301 (cf. Fig. 16)

In 50 μl of Y-100 buffer, there was dissolved 6 μg of pCfBA8 obtained in section 1, 10 units of the restriction enzyme Hin dIII and 8 units of BglII were added, and the digestion reaction was carried out at 37°C for 1 hour. From the reaction mixture, there was obtained, by the LGT method, about 0.6 μg of an hG-CSF cDNA-containing DNA fragment of about 1.4 kb (HindIII-BglII fragment).

Then, the following DNA linker was synthesized:



The design of this DNA linker is such that the 11 amino acids from the first amino acid Thr to the 11th amino acid Gln of hG-CSF are missing in the N-terminal amino acid sequence encoded by said linker.

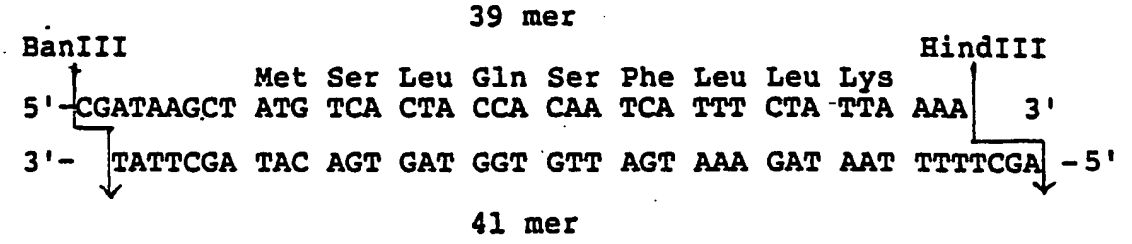
First, the 27-mer and 29-mer single-strand DNAs were synthesized by the ordinary phosphotriester method. The 27-mer and 29-mer (each 2 μg) were dissolved in a total of 40 μl of T4 kinase buffer, 30 units of T4 polynucleotide kinase (Takara Shuzo) was added, and the phosphorylation reaction was conducted at 37°C for 60 minutes.

Then, 0.1 μg of the pCfBA8-derived BanIII-BglII fragment (about 2.7 kb fragment) and 0.1 μg of the pCfBA8-derived Hin dIII-BglII fragment (about 1.4 kb fragment), each obtained as mentioned hereinabove, were dissolved in 25 μl of T4 DNA ligase buffer, and about 2 picomoles of the above DNA linker was added to the mixture solution. After further addition of 6 units of T4 DNA ligase, the ligation reaction was carried out at 4°C for 18 hours.

The recombinant plasmid mixture thus obtained was used to transform E. coli HB101, and an Ap^r colony was obtained. From cultured cells of this colony, there was recovered the plasmid DNA. Thus was obtained pCfTNS301. The hG-CSF derivative encoded by pCfTNS301 is hereinafter called hG-CSF[Δ1-11S].

(10) Construction of pCfTNS401 (cf. Fig. 16)

The following DNA linker was synthesized:



The design of this DNA linker is such that the 7 amino acids from the first amino acid Thr to the 7th amino acid Ser of hG-CSF are missing in the N-terminal amino acid sequence encoded by the linker.

First, the 39-mer and 41-mer single-strand DNAs were synthesized by the ordinary phosphotriester method. The 39-mer and 41-mer (each 2 μg) were dissolved in a total of 40 μl of T4 kinase buffer, 30 units of T4 polynucleotide kinase (Takara Shsuzo) was added, and the phosphorylation reaction was performed at 37°C for 60 minutes.

Then, 0.1 μg of the pCfBA8-derived BanIII-BglII fragment (about 2.7 kb fragment) and 0.1 μg of the pCfBA8-derived HinIII-BglII fragment (about 1.4 kb fragment), each obtained as described above, were dissolved in 25 μl of T4 DNA ligase buffer, and about 2 picomoles of the above-mentioned DNA linker was added to this mixture solution. After further addition of 6 units of T4 DNA ligase, the ligation reaction was conducted at 4°C for 18 hours.

The recombinant plasmid mixture thus obtained was used to transform E. coli HB101, and an Ap^r colony was obtained. The plasmid DNA was recovered from this colony. Thus was obtained pCfTNS401. The hG-CSF derivative encoded by pCfTNS401 is hereinafter called hG-CSF[Δ1-7S].

(11) Construction of pCfTNS501 (cf. Fig. 12)

In 40 μl of Y-100 buffer, there was dissolved pCfBA8-obtained in section 1, 10 units of the restriction enzyme Xho I was added, and the digestion reaction was carried out at 37°C for 1 hour. The DNA recovered by phenol-chloroform extraction and ethanol precipitation was dissolved in 30 μl of Klenow buffer, 2 units of DNA polymerase I Klenow fragment was added, and the reaction was carried out at 17°C for 30 minutes. The DNA polymerase I Klenow fragment was inactivated by 10-minute treatment at 68°C, KCl was added to a concentration of 150 mM, 8 units of the restriction enzyme PvuI was added, and the digestion reaction was conducted at 37°C for 1 hour. From the reaction mixture, there was obtained, by the LGT method, about 2 μg of an hG-CSF cDNA-containing DNA fragment of about 3 kb [XhoI(blunt)-PvuI].

Separately, 5 μg of the ATG vector pTrS20 (3.8 kb) obtained by the procedure of Reference Exlample 4 was dissolved in 50 μl of Y-0 buffer (Y-100 buffer minus 100 mM NaCl), 16 units of the restriction enzyme SacI was added, and the cleavage reaction was carried out at 37°C for 3 hours. The DNA recovered by phenol-chloroform extraction and ethanol precipitation was dissolved in 30 μl of Klenow buffer, 2 units of DNA polymerase I Klenow fragment was added, and the reaction was conducted at 17°C for 30 minutes. The DNA polymerase I Klenow fragment was inactivated by treatment at 68°C for 10 minutes, KCl was added to a concentration of 150 mM, 8 units of the restriction enzyme PvuI was added, and the digestion reaction was performed at 37°C for 1 hour. From the reaction mixture, there was obtained, by the LGT method, about 0.5 μg of a Ptrp-containing DNA fragment of about 1 kb [SacI(blunt)-PvuI].

Then, 0.1 μg of the pCfBA8-derived XhoI(blunt)-PvuI fragment (about 3 kb) and 0.2 μg of the pTrS20-derived SacI(blunt)-PvuI fragment (about 1 kb) were dissolved in 25 μl of T4 DNA ligase buffer, 3 units of T4 DNA ligase was added, and the ligation reaction was carried out at 4°C for 18 hours.

The reaction mixture was used to transform E. coli HB101, an Ap^r colony was obtained, and the plasmid DNA was recovered by the above-mentioned method of Birnboim et al. Thus was obtained pCfTNS501 shown in Fig. 12.

In the hG-CSF derivative encoded by pCfTNS501, the 1st to 6th N-terminal amino acids of mature hG-CSF are missing and the 17th amino acid is Ser in place of Cys. The hG-CSF derivative encoded by pCfTNS501 is hereinafter called hG-CSF[Δ1-6S].

Example 7

(1) Construction of pCfCB101, pCfCC52, pCfCC59, pCfCD28 and pCfCD56 (cf. Fig. 8)

First, 3 μg of pBR322 [Bolivar et al.: Gene, 2, 95 (1977)] was dissolved in 40 μl of Y-100 buffer, 5 units of the restriction enzyme PstI was added, and the digestion reaction was conducted at 37°C for 1 hour. The DNA recovered by phenol-chloroform extraction and ethanol precipitation was dissolved in 20 μl of a solution containing 33 mM Tris-acetic acid (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 5 mM dithiothreitol, and dATP, dCTP, dGTP and dTTP (each 0.4 mM) (hereinafter referred to as "T4 DNA polymerase buffer"), 1 unit of T4 DNA polymerase (Takara Shuzo) was added, and the reaction was carried at 37°C for 30 minutes. The DNA recovered by phenol-chloroform extraction and ethanol precipitation was dissolved in 20 μl of T4 DNA ligase buffer. To this was added about 8 picomoles of the BglII linker DNA [Takara Shuzo; d(pC-A-G-A-T-C-T-G)]. After further addition of 6 units of T4 DNA ligase, the ligation reaction was performed at 4°C for 18 hours. The DNA recovered by phenol-chloroform extraction and

ethanol precipitation was dissolved in 40 µl of Y-100 buffer, 10 units of the restriction enzyme EcoRI and 8 units of BglII were added, and the digestion reaction was conducted at 37°C for 1 hour. From the reaction mixture, there was obtained, by the LGT method, about 0.9 µg of a tetracycline resistance gene portion-containing DNA fragment of about 3.6 kb (EcoRI-BglII fragment).

Separately, 3 µg of pCfBB101 obtained in Example 6-(2), pCfBC52 or pCfBC59 obtained in Example 6-(3) or pCfBD28 or pCfBD56 obtained in Example 6-(4) was dissolved in 10-fold concentrated Y-100 buffer, 5 units of the restriction enzyme EcoRI and 6 units of BglII were added, and the digestion reaction was performed at 37°C for 1 hour. From the reaction mixture, there was obtained, by the LGT method, about 0.4 µg of an hG-CSF cDNA portion-containing DNA fragment of about 1.8 kb (EcoRI-BglII fragment) in each case.

Five tubes each containing a solution of about 0.05 µg of the pBR322-derived EcoRI-BglII fragment (about 3.6 kb) obtained as described above in 20 µl of T4 DNA ligase buffer were prepared. To the tubes were added about 0.1 µg of the pCfBB101-, pCfBC52-, pcfBC59-, pCfBD28-or pCfBD56-derived EcoRI-BglII fragment (about 1.8 kb fragment) and, following further addition of 4 units of. T4 DNA ligase, the ligation reaction was conducted at 4°C for 18 hours.

The recombinant plasmid mixtures obtained were used to transform E. coli HB101, and Tc^r colonies were obtained. From cultured cells of each of these colonies, the plasmid DNA was recovered. Thus, were obtained pCfCB101, pCfCC52, pCfCC59, pCfCD52 and pCfCD56, each shown in Fig. 8. The amino acid sequences of the hG-CSF derivatives encoded by these plasmids are identical with the amino acids of the hG-CSF derivatives encoded by pCfBB101, pCfBC52, pCfBC59, pCfBD28 and pCfBD56, respectively.

Example 8

Production and purification of hG-CSF derivatives E. coli W3110 strA-derived transformants (called ECfTL23, ECfTL35, ECfTL38, ECfTL41, ECfTM14, ECfTM17, ECfTM113, ECfBB101, ECfBC42B1, ECfBC45, ECfBC52, ECfBC59, ECfBC76, ECfBC77, ECfBC93, ECfBC95, ECfBC97, ECfBD28, ECfBD56, ECfBD82, ECfTNS7, ECfTAArg4S, ECfTNS301, ECfTNS401, ECfTNS501, ECfBD28A17 and ECfBD28T17) harboring the recombinant plasmids (obtained in Examples 3, 4, 6 and 7) pCfTL23, pCfTL35, pCfTL38, pCfTL41, pCfTM14, pCfTM17, pCfTM113, pCfBB101, pCfBC42Bl, pCfBC45, pCfBC52, pCfBC59, pCfBC76, pCfBC77, pCfBC93, pCfBC95, pCfBC97, pCfBD28, pCfBD56, pCfBD82, pCfTNS7, pCfAArg4S, pCfTNS301, pCfTN401, pCfTNS501, pCfBD28A17 and pCfBD28A17, respectively were each cultivated in LG medium (prepared by dissolving 10 g of Bactotryptone, 5 g of yeast extract, 5 g of NaCl and 1 g of glucose in 1 liter of water and adjusting the pH to 7.0 with NaOH) at 37°C for 18 hours. A 5-ml portion of the culture broth was inoculated into 100 ml of MCG medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% NaCl, 0.5% casamino acids, 1 mM $MgSO_4$, 4 µg/ml vitamin B_1, pH 7.2) containing 25 µg/ml tryptophan and 50 µg/ml ampicillin. After incubation at 30°C for 4-8 hours, 10 µg/ml 3β-indoleacrylic acid (hereinafter IAA), a tryptophan inducer, was added, and incubation was continued for further 2-12 hours. Cells were harvested by subjecting the culture broth to centrifugation at 8,000 rpm for 10 minutes, and washed with 30 mM NaCl-30 mM Tris-HCl buffer (pH 7.5). The washed cells were suspended in 30 ml of the above mentioned buffer and then disrupted by ultra-sonication at 0°C (BRANSON SONIC POWER COMPANY'S SONIFIER CELL DISRUPTOR 200, Output Control 2, 10 minutes). Centrifugation at 9,000 rpm for 30 minutes gave a cell residue mass. Using the method of Marston et al. [F. A. O. Marston et al.: BIO/TECHNOLOGY, 2 , 800 (1984)], the hG-derivative was extracted from the cell residue mass, purified, solubilized and regenerated. The protein quantity was determined by using Nippon Bio-Rad laboratories' protein assy kit (standard assay method) (M. M. Bradford: Anal. Biochem., 72, 248 (1976)].

The G-CSF activity was determined in the following manner. Bone marrow cells were aseptically collected from the femur of male C3H/He mice of 8-12 weeks of age (Shizuoka Laboratory Animal Center) and suspended in α-MEM supplemented with 10% fetal bovine serum (FBS). Nylon wool (Wako Pure Chemical Industries; Nylon Fiber 146-04231) (0.3 g) packed in a column was impregnat4ed with 1.5 ml of this cell suspension (about 5×107 cells), and the reaction was allowed to proceed in a 5% CO_2 incubator at 37°C for 90 minutes. Then, α-MEM warmed to 37°C in advance was passed through the column, and bone marrow cells unadsorbed on the nylon wool were obtained as an effluent fraction. These cells were washed once with α-MEM and adjusted to a predetermined concentration.

Then, the myelopoietic stem cell colony forming ability was determined by the method of Okabe et al. [Okabe, T. et al: Cancer Research, 44, 4503-4506 (1986)]. Thus, 0.2 ml of the marrow cells suspension prepared as above (2×10^6 cells/ml) was admixed with a mixture of 0.2 ml of α-MEM, 0.4 ml of FBS and 0.2 ml of each 2^2-diluted sample. An equal volume (1.0 ml) of 0.6% agar (Difco, Agar purified #0560-01)

warmed to 42°C was admixed with the mixture, and the whole mixture was distributed in 0.5ml portions into dishes of a 24-dish ploate (Munc's Multidish #143982) (5×10^4 cells/dish, n=3). After incubation in a 5% CO_2 incubator at 37°C for 7 days, colonies each comprising not less than 40 cells were counted under a microscope (Olympus ×40). After colony counting, cells were taken out onto a slide glass with care and fixed with an acetone-formalin mixed solution for 30 seconds. After esterase double stain of the cells by the method of Kubota et al. [Kubota, K. et al.: Exp. Hematology, 8, 339-344 (1980)], each colony was identified.

The potency of eaqch sample was calculated on the basis of the result of counting for the 2 fold dilution in the colony formation assay, as follows. The activity which gives half of the maximum colony formation value for intact G-CSF used as the standard was defined as 50 units. The potency (in units) was calculatred by multiplying by 20, inlcusive of the dilution factor for each sample, for conversion to the activity per milliliter. The specific activity was expressed interms of potency per unit weight (mg) of protein, hence in units/mg.

The potencies of intact G-CSF and G-CSF derivatives are shown in Table 3.

Table 3

Strain	Plasmid borne	Plasmid-encoded product	Specific activity (units/mg)	Specific activity ratio
ECfTA1	pCfTA1	G-CSF(intact)	2.2×10^8	1.0
ECfTL38	pCfTL38	G-CSF(Ser^1)	4.0×10^8	1.8
ECfTL41	pCfTL41	G-CSF(Arg^1)	3.7×10^8	1.7
ECfTL23	pCfTL23	G-CSF(Gly^1)	3.1×10^8	1.4
ECfTL35	pCfTL35	G-CSF(Cys^1)	2.9×10^8	1.3
ECfBB101	pCfBB101	G-CSF(NB101)	7.9×10^8	3.6
ECfBC42B1	pCfBC42B1	G-CSF(NC42B1)	5.1×10^8	2.3
ECfBC45	pCfBC45	G-CSF(NC45)	7.0×10^8	3.2
ECfBC52	pCfBC52	G-CSF(NC52)	6.2×10^8	2.8
ECfBC59	pCfBC59	G-CSF(NC59)	5.9×10^8	2.7
ECfBC76	pCfBC76	G-CSF(NC76)	6.2×10^8	2.8
ECfBC77	pCfBC77	G-CSF(NC77)	7.7×10^8	3.5
ECfBC93	pCfBC93	G-CSF(NC93)	9.2×10^8	4.2
ECfBC95	pCfBC95	G-CSF(NC95)	9.5×10^8	4.3
ECfBC97	pCfBC97	G-CSF(NC97)	8.6×10^8	3.9
ECfBD28	pCfBD28	G-CSF(ND28)	7.9×10^8	3.6
ECfBD56	pCfBD56	G-CSF(ND56)	5.1×10^8	2.3
ECfBD82	pCfBD82	G-CSF(ND82)	4.6×10^8	2.1
ECfTM14	pCfTM14	G-CSF(Ser^1, Cys^3)	3.1×10^8	1.4
ECfTM17	pCfTM17	G-CSF(Ser^1, Arg^3)	3.7×10^8	1.7
ECfTM113	pCfTM113	G-CSF(Ser^1, Ser^3)	2.9×10^8	1.3
ECfTNS7	pCfTNS7	G-CSF(Δ1-4S)	7.7×10^8	3.5
ECfTAArg4S	pCfTAArg4S	G-CSF(Arg^4, Ser^{17})	5.7×10^8	2.6
ECfTNS301	pCfTNS301	G-CSF(Δ1-11S)	3.1×10^8	1.4
ECfTNS401	pCfTNS401	G-CSF(Δ1-7S)	5.5×10^8	2.5
ECfTNS501	pCfTNS501	G-CSF(Δ1-6S)	4.4×10^8	2.0
ECfBD28A17	pCfBD28A17	G-CSF(ND28A17)	6.8×10^8	3.1
ECfBD28T17	pCfBD28T17	G-CSF(ND28T17)	5.9×10^8	2.7
ECfTN205	pCfTN205	G-CSF(Δ1-4)	4.2×10^8	1.9

Example 9

Measurement of activities of hG-CSF derivatives against human bone marrow cells

The marrow fluid was collected from the iliac bone of normal humans of 20-30 years age. An equal volume of α-MEM was added to and mixed with the marrow fluid. A 4-ml of the above bone marrow fluid was layered onto 3 ml of Ficoll-Paque solution (Pharmacia Fine Chemicals, specific gravity 1.077) and, after centrifugation at 400 × g for 30 minutes, the cells occurring in the intermediate layer were separated. The cells were washed twice with PBS (prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4 and 0.2 g of KH_2PO_4 in water to make 1 liter of solution) and then suspended in α-MEM supplumented with 10%

of fetal bovine serum (FBS), and the cell concentration was adjusted to at most 2×10^6 cells ml. A 10-ml portion of the cell suspension was placed in a plastic dish (Falcon 3003) and incubated in a 5% CO_2 incubator at 37°C for 90 minutes. Cells unadsorbed in the plastic dish were recovered, washed once with α-MEM and, after adjustment of the concetration to a predetermined level, subjected to human myelopoietic stem cell growth promoting activity and colony formation tests. Thus, 10% FBS-supplemented α-MEM was distributed in 100-μl portions into wells of a 96-well flat microplate (NUNC, 167008). Then, samples of the hG-CSF and hG-CSF derivatives obtained by the method of Example 8 were added in 100-μl portions into wells of the first row. After thorough mixing, 100 μl of each mixture was transferred to a well of the second row for preparing a 2-fold dilution. Doubling dilution was continued in the same manner until the 12th row (n = 3). In a group, α-MEM alone was used as a negative control.

Then, 100 μl (5×10^4 eukaryotic cells) of the bone marrow cell suspention prepared as described above was sowed into each well. Incubation was carried out in a 5% CO_2 incubator at 37°C for 3 days. During the 20-hour period preceding the last 18 hours, 10 μl of 6-^{3}H-thymidine (Amersham Japan, code TRK61, 107 mci/mg) was added. Cells were recovered onto a glass filter using a cell harvester(Labo-Science), dried, and measured for the radioactivity taken up by the cells using a liquid scintillation counter (Packard, Tricarb 3320).

On the other hand, the human myelopoietic stem cell colony formaiton assay and colony identification were perfomed as described in Example 8.

For calculating the potency of each sample, the activity capable of causing formation of one colony was defined as 1 unit. Thus, the Half Max value (half of the maximum take-up value) was determined based on the dose-response curve showing linearity for the results of counting in the doubling dilution series, and the poteincy of each sample was calculated.

The specific activity was expressed in terms of potency per unit weight (mg) of protein, i.e., in units/mg,

The potencies of the intact hG-CSF and hG-CSF derivatives are shown in Table 4.

Table 4

Strain	Plasmid borne	Plasmid-encoded product	Specific activity (units/mg)	Specific activity ratio
ECfTA1	pCfTA1	G-CSF(intact)	2.8×10^8	1.0
ECfBC59	pCfBC59	G-CSF(NC59)	7.7×10^8	2.8
ECfBC93	pCfBC93	G-CSF(NC93)	7.0×10^8	2.5
ECfBC95	pCfBC95	G-CSF(NC95)	9.5×10^8	3.4
ECfBD28	pCfBD28	G-CSF(ND28)	10.4×10^8	3.7
ECfTAArg4	pCfTAArg4	G-CSR(Arg4)	5.3×10^8	1.9
ECfTNS501	pCfTNS501	G-CSF(Δ1-6S)	6.2×10^8	2.2

Example 10

Production of hG-CSF derivative lacking the N-terminal 1st to 7th amino acids and having serine as the 17th amino acid (hereinafter referred to as M-7S)

To 50 ml of 10 mM Tris-HCl-100 mM NaCl solution (pH 8.0) containing the derivative (a) shown in Table 2 (132 μg/ml) as obtained by cultivating the E. coli strain (ECfBC59) carrying the recombinant plasmid pCfBC59 obtained in Example 6, followed by purification, there was added 0.7 μg of subtilisin BPN'

(8.5 units/mg protein) (Sigma), and incubation was performed at 25°C for 40 hours. After 3-fold dilution with 10 mM Tris-HCl (pH 8.0), the incubation mixture was applied to a DEAE-Toyopearl 650M (Toyo Soda Manufacturing) column (1.7 cm × 4.4 cm) filled with 10 mM Tris-HCl (pH 8.0) at a flow rate of 10 ml/hour. Then, 20 ml of 10 mM Tris-HCl (pH 8.0) was passed through the column at a flow rate of 5 ml/hour. Thereafter, elution was conducted with a buffer system of 10 mM Tris-HCl showing a linear NaCl concentration gradient from 0 M to 0.4 M at the same flow rate (total eluent volume 50 ml). The M-7S derivative was eluted at NaCl concentrations of 100-150 mM (yield 0.7 mg, or 10%). The purity was not less than 90%.

Example 11

Production of M-75

To 50 ml of a 10 mM Tris-HCl-100 mM NaCl solution (pH 8.0) containing the derivative (b) shown in Table 2 132 µg/ml as obtained by cultivatin the E. coli strain (ECfBC59) carrying the recombinant plasmid pCfBC59 obtained in example 6, followed by purification, there was added 0.7 µg of subtilisin BPN' (8.5 units/mg protein) (Sigma), and incubation was performed at 25°C for 14 hours. After 3 fold dilution mixture was applied to DEAE-Toyoperal 650M (Toyo Soda Manufacturing) column (1.7 cm × 4.4 cm) filled with 10 mM Tris-HCl (pH 8.0) at a flow rate of 10 ml/hour. Then, 20 ml of 10 mM Tris-HCl (pH 8.0) was passed through the column at a flow rate of 5 ml/hour. Thereafter, elution was conducted from 0 M to 0.4 M at the same flow rate (total eluent volume 50 ml). The M-7S derivative was eluted at NaCl concentration of 100-150 mM (yield 4.2 mg, or 63%). The purity was not less than 90%.

Example 12

Production of M-7S

To 50 ml of a 10 mM Tris-HCl-100 mM NaCl solution (pH 8.0) containing the derivative (d) shown in Table 2 (132 µg/ml) as obtained by cultivating then E. coli strain (ECfTAArg4) carrying the recombinant plasmid pCfTAArg4 obtained in Example 6, followed by purification, there was added 0.7 µg of subtilisin BPN' (8.5 units/mg protein) (Sigma), and incubation was performed at 25°C for 40 hours. After adjustment of the NaCl concentration to 0.5 M, the incubation mixture was applied to a Zn chelate-Sepharose (Pharmacia Fine Chemicals) column (1.7 cm × 2.6 cm) filled with 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl at a flow rate of 6 ml/hour. Then, 12 ml of 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl was passed through the column at a flow rate of 3 ml/hlur. Thereafter, elution was carried out with a total volume of 30 ml of 10 mM Tris HCl-0.5 M NaCl buffer on a linear pH gradient of from 8.0 to 6.0 at the same rate of flow. The M-7S derivative was eluted in the neighborhood of pH 7.0 (yield 0.6 mg, or 9%). The purity was not less than 90%.

Example 13

Production of hG-CSF derivative lacking the N-terminal 1st to 6th amino acids and having serine as the 17th amino acid (hereinafter referred to as M-6S)

To 50 ml of a solution of the derivative (a) shown in Table 2 (132 µg/ml) in 10 mM Tris-HCl-100 mM NaCl (pH 8.0), there was added 0.7 µg of subtilisin BPN' (8.5 units/mg protein) (Sigma), and incubation was carried out at 25°C for 2 hours. After 3-fold dilution with 10 mM Tris-HCl (pH 8.0), the incubation mixture was applied to a DEAE-Toyopearl 650M (Toyo Soda) column (1.7 cm × 4.4 cm) filled with 10 mM Tris-HCl (pH 8.0) at a flow rate of 10 ml/hour. Then, 20 ml of 10 mM Tris-HCl (pH 8.0) was passed through the column at a flow rate of 5 ml/hour. Thereafter, elution was carried out with a total volume of 50 ml of a buffer system of 10 mM Tris-HCl (pH 8.0) showing a linear NaCl concentration gradient from 0 M to 0.4 M at the same rate of flow. The M-6S derivative was eluted at NaCl concentrations of 100-150 mM (yield 2.6 mg, or 40%). The purity was not less than 90%.

Example 14

Production of M-6S

To 50 ml of a 10 mM Tris-HCl-100 mM NaCl solution (pH 8.0) containing the derivative (a) shown in Table 2 (132 µg/ml), there was added 0.7 µg of Epolozyme (4120 units/mg protein) (Kyowa Hakko Kogyo), and incubation was carried out at 25°C for 20 hours. After adjustment of the NaCl concentration to 0.5 M, the incubation mixture was applied to a Zn chelate-Sepharose (Pharmacia Fine Chemicals) column (1.7 cm × 2.6 cm) filled with 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl at a flow rate of 6 ml/hour. Then, 12 ml of 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl was passed through the column at a flow rate of 3 ml/hour. Thereafter, elution was carried out with a total volume of 30 ml of 10 mM Tris-HCl-0.5 M NaCl buffer on a linear pH gradient of from 8.0 to 6.0 at the same rate of flow. The M-6S derivative was eluted in the neighborhood of pH 7.0 (yield 2.5 mg, or 38%). The purity was not less than 90%.

Example 15

Production of M-6S

To 50 ml of a 10 mM Tris-HCl-100 mM NaCl solution (pH 8.0) containing the derivative (b) shown in Table 2 (132 µg/ml), there was added 0.7 µg of subtilisin amylosacchalyticus, and incubation was carried out at 25°C for 20 hours. After adjustment of the NaCl concentration to 0.5 M, the incubation mixture was applied to a Zn chelate-Sepharose (Pharmacia Fine Chemicals) column (1.7 cm × 2.6 cm) filled with 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl at a flow rate of 6 ml/hour. Then, 12 ml of 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl was passed through the column at a flow rate of 3 ml/hour. Thereafter, elution was carried out with a total volume of 30 ml of 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl buffer on a linear pH gradient of from 8.0 to 6.0 at the same rate of flow. The M-6S derivative was eluted in the neighborhood of pH 7.0 (yield 2.5 mg, or 38%). The purity was not less than 90%.

Example 16

Production of M-6S

To 50 ml of a 10 mM Tris-HCl-100 mM NaCl solution (pH 8.0) containing the derivative (d) shown in Table 2 (132 µg/ml), there was added 0.7 µg of subtilisin Carlsberg (0.034 unit/mg protein) (NOVO), and incubation was carried out at 25°C for 20 hours. After adjustment of the NaCl concentration to 0.5 M, the incubation mixture was applied to a Zn chelate-Sepharose (Pharmacia Fine Chemicals) column (1.7 cm × 2.6 cm) at a flow rate of 6 ml/hour. Then, 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl was passed through the column at a flow rate of 3 ml/hour. Thereafter, elution was carried out with a total volume of 30 ml of a buffer system of 10 mM Tris-HCl-0.5 M NaCl showing a linear pH gradient of from 8.0 to 6.0 at the same rate of flow. The M-6S derivative was eluted in the neighborhood of pH 7.0 (yield 3 mg, or 45%). The purity was not loess than 90%.

Example 17

Production of M-6S

To 50 ml of a 10 mM Tris-HCl-100 mM NaCl solution (pH 8.0) containing the derivative (a) shown in Table 2 (132 µg/ml), there was added 0.7 µg of proteinase K (0.027 unit/mg protein) (Sigma), and incubation was carried out at 25°C for 40 hours. After 3-fold dilution with 10 mM Tris-HCl (pH 8.0), the incubation mixture was applied to a DEAE-Toyopearl 650M (Toyo Soda) column (1.7 cm × 4.4 cm) filled with 10 mM Tris-HCl (pH 8.0) at a flow rate of 10 ml/hour. Then, 20 ml of 10 mM Tris-HCl (pH 8.0) was passed through the column at a flow rate of 5 ml/hour. Thereafter, elution was carried out with a total volume of 50 ml of a buffer system of 10 mM Tris-HCl (pH 8.0) showing a linear NaCl concentration gradient of from 0 M to 0.4 M at the same rate of flow. The M-6S derivative was eluted at NaCl concentrations of 100-150 mM (yield 2.6 mg, or 39%). The purity was not less than 90%.

Example 18

Production of hG-CSF derivative lacking the N-terminal 1st to 5th amino acids and having serine as the 17th amino acid (hereinafter referred to as M-5S)

To 50 ml of a 10 mM Tris-HCl-100 mM NaCl solution (pH 8.0) containing the derivative (b) shown in Table 1 (132 µg/ml), there was added 0.5 µg of trypsin (267 units/mg protein) (Sigma), and incubation was carried out at 25°C for 10 hours. After adjustment of the NaCl concentration to 0.5 M, the incubation mixture was applied to a Zn chelate-Sepharose (Pharmacia Fine Chemicals) column (1.7 cm × 2.6 cm) filled with 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl at a flow rate of 6 ml/hour. Then, 12 ml of 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl was passed through the column at a flow rate of 3 ml/hour. Thereafter, elution was carried out with a total volume of 30 ml of a buffer system of 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl showing a linear imidazole concentration gradient of from 0 M to 0.3 M at the same rate of flow. The M-5S derivative was eluted at 0.1 M imidazole (yield 2.7 mg, or 41%). The purity was not loess than 90%.

Example 19

Production of hG-CSF derivative lacking the N-terminal 1st to 4th amino acids and having serine as the 17th amino acid (hereinafter referred to as M-4S)

To 50 ml of a 10 mM Tris-HCl-100 mM NaCl solution (pH 8.0) containing the derivative (d) shown in Table 1 (132 µg/ml), there was added 5 µg of trypsin (267 units/mg protein) (Sigma), and incubation was carried out at 25°C for 20 hours. After adjustment of the NaCl concentration to 0.5 M, the incubation mixture was applied to a Zn chelate-Sepharose (Pharmacia Fine Chemicals) column (1.7 cm × 2.6 cm) filled with 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl at a flow rate of 6 ml/hour. Then, 12 ml of 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl was passed through the column at a flow rate of 3 ml/hour. Thereafter, elution was carried out with a total volume of 30 ml of a buffer system of 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl showing a linear imidazole concentration gradient of from 0 M to 0.3 M at the same rate of flow. The M-4S derivative was eluted at 0.1 M imidazole (yield 2.7 mg, or 41%). The purity was not less than 90%.

Example 20

Production of M-4S

To 50 ml of a 10 mM Tris-HCl-100 mM NaCl solution (pH 8.0) containing the derivative (d) shown in Table 2 (132 µg/ml), there was added 5 µg of α-chymotripsin (267 units/mg protein) (Sigma), and incubation was carried out at 25°C for 20 hours. After adjustment of the NaCl concentration to 0.5 M, the incubation mixture was applied to a Zn chelate-Sepharose (Pharmacia Fine Chemicals) column (1.7 cm × 2.6 cm) filled with 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl at a flow rate of 6 ml/hour. Then, 12 ml of 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl was passed through the column at a flow rate of 3 ml/hour. Thereafter, elution was carried out with a total of 30 ml of a buffer system of 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl showing a linear imidazole concentration gradient of from 0 M to 0.3 M at the same rate of flow. The M-4S derivative was eluted at 0.1 M imidazole (yield 2.3 mg, or 35%). The purity was not less than 90%.

Example 21

As seen in Examples 10-20 there can be obtained those hG-CSF derivatives which have serine as a substitute for the 17th amino acid and are lacking in 4 (M-4S), 5 (M-5S), 6 (M-6S) and 7 (M-7S)N-terminal amino acids. The use of recombinant DNA technology generally results in addition of methionine to the N terminus, and this is one of the disadvantageous features of recombinant products. On the contrary, the use of the enzymatic cleavage technique according to the invention is advantageous since such products can be produced without addition of methionine to the N terminus.

The derivatives obtained in this manner were assayed for G-CSF activity for comparison. The results obtained are shown in Table 5.

Table 5

Activity comparison among G-CSF derivatives formed by the enzymatic cleavage technique

hG-CSF Derivative	Relative Activity (derivative/intact)
Intact	1.0
M-4S	4.0
M-5S	3.5
M-6S	3.0
M-7S	3.3

From the results shown in Table 5, it was found that, in the above in vitro evaluation, the derivatives lacking in 4-7 N-terminal side amino acids have a 2-to 4-fold higher activity as compared with the intact hG-CSF.

Therefore, the derivatives lacking in N-terminal side amino acids that can be produced in accordance with the present invention have no methionine added to the N terminus and are 2-to 4-fold higher in activity than the intact product.

The following examples illustrate the acquisition of reactivity (susceptibility) to cleavage by hydrolytic enzymes as a result of mutation in the N-terminal portion.

Test Example 1

Comparison in reactivity with subtilisin Carlsberg between intact hG-CSF and N-terminal mutants of hG-CSF

The derivatives shown in Table 2 and intact hG-CSF were each incubated in the presence of 3.6×10^4 units/mg G-CSF of subtilisin Carlsberg (NOVO) at 25°C for 14 hours in the same manner as in Example 16. While the derivatives shown in Table 2 gave the M-6S derivative, the intact hG-CSF remained unreacted. Furthermore, even when this enzyme was used in a 100-fold increased amount (3.6×10^2 units/mg G-CSF), the intact product failed to produce M-6S but preferentially underwent global decomposition reactions.

Test Example 2

Comparison in reactivity with trypsin between intact hG-CSF and N-terminal mutants of hG-CSF

The derivative (a) shown in Table 2 and intact hG-CSF were each incubated in the presence of 0.22 units/mg G-CSF of trypsin (Sigma) at 25°C for 20 hours. While the derivative (a) shown in Table 2 gave the M-4S derivative, the intact hG-CSF remained unreacted Furthermore, even when the enzyme was in a 100-fold increased amount (22 units/mg G-CSF), the intact hG-CSF did not give M-4S but preferentially underwent global decomposition reactions.

Test Example 3

Heat stability of hG-CSF derivatives

A 20-μg portion of each of the various derivatives, shown in Table 6, of the invention was dissolved in 1 ml of phosphate-buffered physiological saline (PBS) (pH 7.2) or α-MEM supplemented with 10% fetal bovine serum (FBS). Incubation was carried out at 56°C, and samples were collected at timed intervals and assayed for CSF activity by colony formation testing using mouse bone marrow cells (the above-mentioned method of Okabe et al.).

Each sample was diluted by the doubling dilution technique from 40 ng/ml to give 10 dilution levels. For each level, activity assay was performed, and the residual activity was determined by comparing the activity at a certain concentration at which good dose-response with that before heating (0 minute).

The residual activity (corresponding to thermal stability) data obtained in PBS and in 10% FBSsupplemented α-MEM are shown in Table 6(A) and Table 6(B), respectively.

Table 6 (A)

	Residual Activity (%)		
Sample	30 min.	60 min.	120 min.
Intact G-CSF	45.2	16.4	12.7
NC93	98.0	93.3	90.6
ND28	68.7	52.8	33.9
Arg4	33.8	15.0	12.9
M-7S	84.8	72.0	57.0
1-4S	89.7	72.4	61.6

Table 6 (B)

	Residual activity (%)		
Sample	30 min.	60 min.	120 min.
Intact G-CSF	9.1	6.7	8.9
ND28	55.6	46.5	32.4
NC59	45.1	35.6	24.9

Example 22

Construction of pCfBD28A17 and pCfBD28T17 using site-specific mutagenesis (cf. Fig. 17)

(a) Construction of single-strand template DNA (single-strand pt19BD28N)

In 50 µl of Y-100 buffer, there was dissolved 3 µg of pCfBD28 obtained by the procedure of Example 6-(4), 10 units each of the restriction enzymes BanIII (Toyobo) and PstI were added, and the cleavage reaction was carried out at 37°C for 2 hours. From the reaction mixture, there was obtained by the LGT method, about 0.1 µg of an about 210 bp DNA fragment (Ban III-PstI fragment) coding for the N-terminal portion of the hG-CSF derivative (ND28).

Separately, 1 µg of the M13 phage vector M13mp19RF DNA (Takara Shuzo) was dissolved in a total of 50 µl of Y-50 buffer, 10 units of the restriction enzyme AccI (Toyobo) was added, and the cleavage reaction was carried out at 37°C for 2 hours. Thereafter, NaCl was added to an NaCl concentration of 100 mM, 10 units of the restriction enzyme PstI was added, and the cleavage reaction was conducted at 37°C for 2 hours. From the reaction mixture, there was obtained, by the LGT method, about 0.8 µg of a DNA fragment of about 7.24 kb (Acc I-PstI fragment).

In 50 µl of T4 DNA ligase buffer, there were dissolved 0.2 µg of the BanIII-PstI fragment (about 210 bp) and 0.05 µg of the AccI-PstI fragment (about 7.24 kb), each obtained as described above. T4 DNA ligase (10 units) was added to the mixture solution, and the ligation reaction was performed at 12°C for 16 hours.

Then, the above reaction mixture was used to transfect E. coli JM105 by a known method. Thus was obtained a recombinant phage. From cultured cells of an E. coli JM105-derived transformant infected with the recombinant phage, there was recovered the recombinant M13 phage RF DNA. The structure of this RF DNA (hereinafter referred to as pt19BD28N) was confirmed by cleavage with PstI, EcoRI, AvaI and XhoI followed by polyacrylamide gel electrophoresis. Then, the single-strand pt19BD28N was recovered from the recombinant phage by a known method and used as an template.

(b) Construction of gapped duplex DNA

In 30 µl of Y-100 buffer, there was dissolved 3 µg of the M13mp19 RF DNA (Takara Shuzo), 10 units each of the restriction enzymes EcoRI and HindIII were added, and the cleavage reaction was carried out at 37°C for 2 hours. From the reaction mixture, there was obtained, by the LGT method, about 2.5 µg of a DNA fragment of about 7.2 kb (EcoRI-HindIII fragment).

This M13mp19 RF DNA-derived EcoRI-HindIII fragment (about 7.2 kb) and 1 µg of the single-strand template DNA pt19BD28N obtained as described in the preceding section were dissolved in 27 µl of Klenow buffer, and DNA denaturation was caused by boiling at 100°C for 6 minutes. Thereafter, the mixture was allowed to stand at 65°C for 10 minutes, at 37°C for 40 minutes, at 4°C for 40 minutes and in ice for 10 minutes to cause the annealing reaction to proceed, whereby a gapped duplex DNA was formed in which the G-CSF gene portion alone in the template was single-stranded. The thus-formed gapped duplex DNA was recovered by the LGT method.

(c) Mutagenesis (construction of pt19BD28NA17 and pt19BD28NT17)

A single-strand DNA (D-1) required for substituting A1a for the 17th amino acid (from the N terminus), namely Ser, of the hG-CSF derivative [ND28] obtained in Example 6 and a single-strand DNA (D-2) required for substituting Thr for the Ser were synthesized by the ordinary phosphotriester method. The base sequences of D-1 (33-mer) and D-2 (33-mer) are shown below:

```
D-1                                   Ala
                                       ↑
                 12  13  14  15  16  17  18  19  20  21  22
 17              Ser Phe Leu Leu Lys Ser Leu Glu Gln Val Arg
(Ser→Ala) 5'-AGC TTC CTT TTA AAG GCC TTA GAG CAA GTG AGG
                                    StuI          -3' (33 mer)
```

D-2

```
                                        Thr
                                         ↑
                 12  13  14  15  16  17  18  19  20  21  22
  17             Ser Phe Leu Leu Lys Ser Leu Glu Gln Val Arg
(Ser→Thr) 5'-AGC TTC CTT TTA AAA ACT CTA GAG CAA GTG AGG
                                     XbaI     -3' (33 mer)
```

The designs of the above DNAs are such that mutagenesis using D-1 can cause formation of a new StuI site and mutagenesis using D-2 can give rise to a new XbaI site. Therefore, mutants can be identified by cleavage with these restriction enzymes.

D-1 and D-2 were each individually dissolved, in an amount of 1 μg, in 50 μl of T4 kinase buffer, 30 units of T4 polynucleotide kinase was added, and the phosphorylation reaction was carried out at 37°C for 60 minutes.

Then, 0.2 μg of the phosphorylated D-1 or D-2 and 0.1 μg of the gapped duplex DNA obtained as described in the opening section were dissolved in 34 μl of buffer containing 6.5 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 100 mM NaCl, the solution was allowed to stand at 65°C for 60 minutes and then at room temperature for 30 minutes, whereby D-1 or D-2 was annealed with the gapped duplex DNA.

To the solution were added dATP, dTTP, dCTP and dGTP each to a concentration of 0.5 mM. Following further addition of 1.5 units of DNA polymerase I Klenow fragment and 10 units of T4 DNA ligase, the extension reaction was carried out at 4°C for 16 hours.

The reaction mixture thus obtained were used to transfect E. coli JM105, and mutant phages were obtained. The RF DNAs were recovered from the mutant phage-infected E. coli JM105 transformants and identified by cleavage with AvaI, XhoI and StuI (when D-1 was used) or with XbaI (when D-2 was used), followed by polyacrylamide gel electrophoresis. The RF DNA with mutation introduced therein by means of D-1 is named pt19BD28NA17 and the RF DNA with mutation introduced therein by means of D-2 is named pt19BD28NT17. The base sequences of pt19BD28NA17 and pt19BD28NT17 in the vicinity of the StuI site and XbaI site, respectively, were confirmed by the dideoxy sequencing method using M13 phage to be as follows:

pt19BD28NA17

```
                 12  13  14  15  16  17  18  19  20  21  22
                 Ser Phe Leu Leu Lys Ala Leu Glu Gln Val Arg
                 AGC TTC CTT TTA AAG GCC TTA GAG CAA GTG AGG
                                     ↑
                                    StuI
```

pt19BD28NT17

```
                 12  13  14  15  16  17  18  19  20  21  22
                 Ser Phe Leu Leu Lys Thr Leu Glu Gln Val Arg
                 AGC TTC CTT TTA AAA ACT CTA GAG CAA GTG AGG
                                         ↑
                                        XbaI
```

(d) Construction of pCfBD28A17 and pCfBD28T17

In 50 μl of Y-100 buffer, there was dissolved 3 μg of pt19BD28NA17 or pt19BD28NT17 obtained as described above, 10 units each of the restriction enzymes AvaI and XhoI were added, and the cleavage reaction was conducted at 37°C for 2 hours. From the reaction mixture, there was obtained, by the LGT method, 0.05 μg of an about 110 bp DNA fragment containing the site of mutation introduced as described in the preceding section (AvaI-XhoI fragment).

Separately, 2 µg of pCfBD28 obtained in Example 6-(4) was dissolved in 50 µl of Y-100 buffer, 10 units each of the restriction enzymes XhoI and BglII were added, and the cleavage reaction was performed at 37°C for 2 hours. From the reaction mixture, there was obtained, by the LGT method, about 1 µg of a tryptophan promoter portion-containing DNA fragment of about 2.74 kb (XhoI-BGlII fragment).

Further, separately, 2 µg of pCfBD28 was dissolved in 50 µl of Y-100 buffer, 10 units of the restriction enzyme BglII was added, and the cleavage reaction was carried out at 37°C for 2 hours. After confirmation of the completeness of the BglII cleavage by agarose gel electrophoresis, 5 units of the restriction enzyme AvaI was added, and partial cleavage was effected at 37°C for 10 minutes. From the reaction mixture, there was obtained, by the LGT method, 0.4 µg of an about 1.29 kb DNA fragment (BglII-AvaI fragment) containing most of the mature hG-CSF cDNA with the *t*pp terminator portion.

Then, 0.1 µg of the pCfBD28-derived XhoI-BglII fragment (about 2.74 kb), the pCfBD28-derived BglII-AvaI fragment (about 1.29 kb) and 0.02 pg of the pt19BD28NA17-or pt19BD28NT17-derived AvaI-XhoI fragment (about 110 bp) were dissolved in 60 µl of T4 DNA ligase, 10 units of T4 DNA ligase was added, and the ligation reaction was conducted at 12°C for 16 hours.

The reaction mixture thus obtained was used to transform E. coli HB101, and an Ap^r colony was obtained. The plamid DNA was recovered from cultured cells of this colony. The plasmid constructed by using pt19BD28NA17 is named pCfBD28A17 and that constructed by using pt19BD28NT17 is named pCfBD28T17. The structure of pCfBD28A17 was confirmed by cleavage with AvaI, XhoI, BglII and StuI, followed by agarose gel electrophoresis. The structure of pCfBD28T17 was confirmed by cleavage with AvaI, XhoI, BglII and XbaI, followed by agarose gel electrophoresis.

The replacing amino acid residues in the hG-CSF derivatives encoded by these two plasmids as compared with mature hG-CSF are as follows:

Position of amino acid substitution (amino acid of hG-CSF)	Plasmid	
	pCfBD28A17	pCfBD28T17
1st (Thr)	Ala	Ala
3rd (Leu)	Thr	Thr
4th (Gly)	Tyr	Tyr
5th (Pro)	Arg	Arg
17th (Cys)	Ala	Thr

The hG-CSF derivatives encoded by pCfBD28A17 and pCfBD28T17 are hereinafter called hG-CSF-[ND28A17] and hG-CSF[ND28T17], respectively.

Reference Example 1

Isolation of the hG-CSF cDNA-carrying plasmid pCSF1-2

(1) Preparation of poly(A) RNA from normal human peripheral blood macrophage

Macrophages, which are adherent cells, were isolated by culturing leukocytes obtained by centrifugation of normal human peripheral blood in a plastic bottle and removing nonadherent cells by washing. An RNA having poly(A) was prepared from the macrophages by the guanidine thiocyanate-lithium chloride method [Cathala et al.: DNA, 2, 329 (1983)], as follows.

Normal human peripheral blood (400 ml) was centrifuged on a Hitachi RPR10 rotor at 1,800 rpm for 20 minutes. The resultrant blood cell precipitate was suspended in 50 ml of phosphate-buffered saline [8 g/liter NaCl, 0.2 g/liter KCl, 1.15 g/liter anhydrous Na_2HPO_4, 0.2 g/liter KH_2PO_4 (pH 7.2); hereinafter abbreviated as PBS]. A 25-ml portion of this suspension was layered on 25 ml of lymphocyte separation liquid (BIONETICS), and the whole was centrifuged on a Hitachi RPR10 rotor at 1,800 rpm for 30 minutes. Leukocytes in the middle layer were collected, washed with an equal volume of PBS (on a Hitachi RPR10

rotor at 1,500 rpm for 10 minutes), then suspended in 20 ml of RPMI 1640 medium (Nissui Seiyaku) containing 5% fetal bovine serum, and cultured using a tissue culture flask (Corning). After growing at 37°C for 1.5 hours, the culture supernatant was removed together with nonadherent cells. A fresh 20-ml portion of the same medium and E. coli-derived lipopolysaccharide (LPS) (in an amount to give a concentration of 0.3 mg/ml) were added, and cultivation was continued at 37°C for further 4 hours. Then, cells were harvested from the culture by centrifugation at 1,100 × g at 4°C for 10 minutes, washed with 80 ml of PBS and solubilized in 10 ml of a solution comprising 5 M guanidine thiocyanate, 10 mM EDTA, 50 mM Tris-HCl (pH 7) and 8% (v/v) 2-mercaptoethanol using a vortex mixer. This solubilization product was transferred to a centrifuge tube, 80 ml of 4 M LiCl was added, and the mixture was stirred, then allowed to stand at 4°C for 20 hours and centrifuged on a Hitachi RPR10 rotor at 9,000 rpm for 90 minutes. Thereafter, an RNA precipitate was recovered. The RNA precipitate was suspended in 50 ml of a solution comprising 4 M urea and 2 M lithium chloride, and the suspension was centrifuged on a Hitachi RPR10 rotor at 9,000 rpm for 60 minutes, and an RNA precipitate was again recovered.

The RNA precipitate was dissolved in 10 ml of a solution comprising 0.1% sodium lauryl sulfate, 1 mM EDTA and 10 mM Tris-HCl (pH 7.5) and the RNA was recovered by phenol-chloroform extraction and ethanol precipitation. The RNA obtained (about 0.8 mg) was dissolved in 1 ml of a solution comprising 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. After incubation at 65°C for 5 minutes, 0.1 ml of 5 M NaCl was added. The mixture was subjected to oligo(dT)cellulose column (P-L Biochemicals) chromatography (column volume 0.5 ml). The adsorbed, poly(A)-containing mRNA was eluted with a solution comprising 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA to give about 30 μg of poly(A)-containing mRNA.

(2) cDNA synthesis and insertion of the DNA into a vector

The Okayama-Berg method [Mol. Cell. Biol., 2, 161 (1982)] was used for cDNA synthesis and recombinant plasmid construction by insertion of the cDNA obtained. The processes therefor are outlined in Fig. 9.

To 300 μl of a solution comprising 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 10 mM NaCl, there was added 400 μg of pCDV1 [Okayama & Berg: Mol. Cell. Biol., 3, 280 (1983)] and, after further addition of 500 units of Kpn I, the reaction was carried out at 37°C for 6 hours, whereby the plasmid was cleaved at the KpnI site. The DNA was recovered by phenol-chloroform extraction and ethanol precipitation. About 200 μg of the KpnI-cleaved DNA was added to 200 μl of a solution prepared by adding dTTP in a concentration of 0.25 mM to a buffer (hereinafter abbreviated as TdT buffer) comprising 40 mM sodium cacodylate, 30 mM Tris-HCl (pH 6.8), 1 mM $CaCl_2$ and 0.1 mM dithiothreitol (hereinafter abbreviated as DTT) and, after further addition of 81 units of terminal deoxynucleotidyl transferase (hereinafter abbreviated as TdT) (P-L Biochemicals), the reaction was carried out at 37°C for 11 minutes, whereby a poly(dT) chain comprising about 67 dT residues was added to each KpnI cleavage site 3′ end of pCDV1. About 100 μg of the poly(dT) chain-added pCDV1 DNA was recovered from the above reaction mixture by phenol-chloroform extraction and ethanol precipitation. The DNA was added to 150 μl of a buffer comprising 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 100 mM NaCl, 360 units of EcoRI was further added, and the reaction was carried out at 37°C for 2 hours. The reaction mixture was treated by the LGT method, and a DNA fragment of about 3.1 kb was recovered. Thus was obtained about 60 μg of the poly(dT) chain-tailed pCDV1. The DNA was dissolved in 500 μl of a solution comprising 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, the solution was incubated at 65°C for 5 minutes and then cooled with ice, and 50 μl of 5 M NaCl was added. The mixture was subjected to oligo(dA)-cellulose column (Collaborative Research) chromatography. Molecules having a sufficient poly-(dT) chain length were adsorbed on the column and they were eluted with a solution comprising 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Thus was obtained 27 μg of the poly(dT) chain-tailed pCDV1 (hereinafter abbreviated as vector primer).

Then, a linker DNA was synthesized.

To 200 μl of a buffer comprising 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 50 mM NaCl, there was added about 14 μg of pL1 [Okayama & Berg: Mol. Cell. Biol., 3, 280 (1983)], 50 units of PstI was further added, and the reaction was carried out at 37°C for 4 hours, whereby the pL1 DNA was cleaved at the PstI site. The reaction mixture was subjected to phenol-chloroform extraction, followed by ethanol precipitation, whereby about 13 μg of the PstI-cleaved pL1 DNA was recovered. The DNA (about 13 μg) was added to 50 μl of TdT buffer supplemented with dGTP in a final concentration of 0.25 mM, 54 units of TdT (P-L Biochemicals) was further added, and the mixture was incubated at 37°C for 13 minutes, whereby a (dG) chain containing about 14 dG residues was added to pL1 at each 3′ end at the PstI cleavage site. The DNA was recovered by phenol-chloroform extraction followed by ethanol precipitation. The DNA was added to

100 µl of a buffer comprising 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 60 mM NaCl, 80 units of HindIII was further added, and the mixture was incubated at 37°C for 3 hours, whereby the pL1 DNA was cleaved at the HindIII site. The reaction mixture was fractionated by agarose gel electrophoresis, and a DNA fragment of about 0.5 kb was recovered by the DEAE-paper method [Dretzen et al.: Anal. Biochem., 112, 295 (1981)]. Thus was obtained the oligo(dG) chain-tailed linker DNA (hereinafter referred to simply as linker DNA).

In 22.3 µl of a solution comprising 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 30 mM KCl, 0.3 mM DTT, 2 mM dNTP (dATP, dTTP, dGTP and dCTP) and 10 units of ribonuclease inhibitor (P-L Biochemicals), there were dissolved about 3 µg of the poly(A) RNA and about 1.4 µg of the vector primer, each prepared as described above, 10 units of reverse transcriptase (Seikagaku Kogyo) was added, and the mRNA was caused to synthesize a DNA complementary thereto by incubating the mixture at 41°C for 90 minutes. The reaction mixture was subjected to phenol-chloroform extraction and the vector primer DNA with the RNA-DNA double strand added thereto was recovered by ethanol precipitation. This DNA was dissolved in 20 µl of TdT buffer containing 66 µM dCTP and 0.2 µg of poly(A), 14 units of TdT (P-L Biochemicals) was added, and the mixture was incubated at 37°C for 2 minutes, whereby a (dC) chain containing 20 dC residues was added to the 3′ end of the cDNA. The reaction mixture was extracted with phenol-chloroform, and the (dC) chain-tailed cDNA-vector primer DNA was recovered by ethanol precipitation. The DNA was dissolved in 400 µl of a solution comprising 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 60 mM NaCl, 20 units of HindIII was added, and incubation was conducted at 37°C for 2 hours to cause cleavage at the HindIII site. Phenol-chloroform extraction of the reaction mixture and the subsequent ethanol precipitation gave 0.5 picomole of the (dC) chain-tailed cDNA-vector primer DNA. In 100 µl of a solution comprising 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl and 1 mM EDTA, there were dissolved 0.2 picomole of the DNA and 0.4 picomole of the above-mentioned linker DNA, and incubation was carried out at 65°C, 42°C and 0°C for 10 minutes, 25 minutes and 30 minutes, respectively. A total volume of 1,000 µl of a reaction mixture was prepared which had the following composition: 20 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1 M KCl and 0.1 mM β-NAD. To this reaction medium was added 25 units of E. coli-derived DNA ligase (New England Bio-Labs), and incubation was carried out at 11°C for 18 hours. The reaction medium was supplemented with 40 µM each dNTP and with β-NAD to give a final concentration of 0.15 mM, 10 units of E. coli DNA ligase, 20 units of E. coli DNA polymerase I (P-L Biochemicals) and 10 units of E. coli ribonuclease H (P-L Biochemicals) were added, and incubation was carried out at 12°C for 1 hour and then at 25°C for 1 hour. The above reaction procedure caused circularization of the cDNA-containing recombinant DNA and substitution of the RNA portion of the RNA-DNA double strand by the corresponding DNA. Thus was formed the recombinant plasmid in the completely double-stranded DNA form.

(3) Selection of the hG-CSF cDNA-containing recombinant DNA

The recombinant plasmid obtained as described in (2) was used to transform E. coli C600SF8 by the method of Scott et al. [Katsuya Shigesada: Saibo Kogaku (Cell Technology), 2, 616 (1983)]. About 9,200 colonies obtained were fixed on a nitrocellulose filter. One strain capable of associating strongly at 60°C with a probe prepared by labeling, with ^{32}P, the 27-base synthetic DNA 5′-ACCCCCCTGGGCCCTGCCAGCTCCCTG-3′ corresponding to the N-terminal 9 amino acids of the mature hG-CSF protein as isolated by Nagata et al [Nagata et al.: Nature, 319, 415 (1986)] was selected [the Grunstein-Hogness method; Proc. Natl. Acad. Sci. USA, 72, 3961 (1975)]. The whole base sequence of the cDNA contained in the plasmid pCSF1-2 carried by this strain was determined by the dideoxy sequencing method using M13 phage (Table 1). As a result, it was found that the cDNA contained in pCSF1-2 codes for hG-CSF.

This bacterial strain has been deposited with the FRI under the designation E. coli ECSF1-2 (FERM BP-1220), as mentioned hereinabove.

Reference Example 2

Isolation and purification of the plasmid pKYP26

A pKYP26-bearing E. coli strain [E. coli IKYP26 (FERM BP-863)] was cultured in 10 ml of L medium (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) containing 50 µg/ml of ampicillin at 37°C for 18 hours. The whole culture was transferred to 1 liter of L medium containing 50 µg/ml of amplicillin, and

cultured at 37°C. After 4 hours, chloramphenicol was added in a concentration of 170 μg/ml, and cultivation was continued at 37°C for additional 16 hours. Cells were harvested by centrifugation (5,000 rpm, 10 minutes), washed with 0.8% NaCl and suspended in 20 ml of 50 mM Tris-HCl (pH 8.0), and the suspension was cooled with ice. Lysozyme (10 mg/ml, 8 ml) was added and, after standing in ice for 10 minutes, 9.6 ml of 0.5 M EDTA was added. After standing in ice for 10 minutes, 2.3 ml of 2% Triton X-100 (Wako Pure Chemical Industries) was added, followed by further standing in ice for 1 hour. Ultracentrifugation at 50,000 × g at 4°C for 1 hour gave about 40 ml of a supernatant. Then, this supernatant was adjusted to pH 12.5 by addition of 3 M NaOH and stirred gently at room temperature for 10 minutes. The pH was brought back to 8.5 by addition of 2 M Tris-HCl (pH 7.5), followed by further stirring for 3 minutes. At this timepoint, the liquid volume was about 55 ml. A 1/9 volume of 5 M NaCl was added and then phenol extraction was carried out. A 1/250 volume of 5 mg/ml RNase A (Sigma) was added, the RNA degradation reaction was conducted at 37°C for 1 hour, a 1/5 volume of 5 M NaCl was then added, and a 1/3 volume of 30% PEG 6000 (Nakarai Chemicals) was added. The resultant mixture was allowed to stand at -20°C for 2 hours. The resultant precipitate was collected by centrifugation and dissolved in 2 ml of a solution comprising 10 mM Tris HCl (pH 7.5) and 1 mM EDTA, sodium dodecyl sulfate (SDS) was added in a concentration of 0.5%, proteinase K (Sigma) was added in a concentration of 50 μg/ml, and the proteolytic reaction was carried out at 37°C for 1 hour. After three repetitions of phenol extraction, the DNA was recovered by chloroform extraction and ethanol precipitation, and dissolved in 1 ml of a solution comprising 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. In this way, 800 μg of pKYP26 could be obtained. The structure of pKYP26 was confirmed by cleavage with EcoRI, KpnI, BamHI, BglII and PstI, followed by agarose gel electrophoresis.

Reference Example 3

1) Isolation of the human LT cDNA-carrying plasmid pLT1

(1) Preparation of poly(A) RNA from LukII cells

The guanidine thiocyanate-lithium chloride method [Cathala et al.: DNA, 2 , 329 (1983)] was followed to prepare a poly(A)-carrying RNA from the human lymphoblastoid cell line LukII , as follows:

Human lymphoblastoid LukII cells [Berish Y. Rubin et al.: Proc. Natl. Acad. Sci. USA, 82, 6637 (1985)] were sowed into 1 liter of RPMI 1640 medium (Nissui Seiyaku) containing 5% fetal bovine serum and 1 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES) in a cell concentration of 8×10^5 cells/ml and grown there. A spinner culture bottle was used for the culture. After cultivation at 37°C for 48 hours, cells were collected by centrifugation and transferred to a freshl-liter portion or RPMI 1640 medium containing 5% fetal bovine serum and 1 mM HEPES, and cultivation was conducted at 37°C for further 48 hours. Thenm cells were harvested from a portion (250 ml) of this cell suspension by centrifugation at 1,100 × g at 4°C for 10 minutes, washed with 80 ml of phosphate buffer, and solubilized in 10 ml of a solution comprising 5 M guanidine thiocyanate, 10 mM EDTA, 50 mM Tris-HCl (pH 7) and 8% (v/v) 2-mercaptoethanol using a vortex mixer. The solubilization product was transferred to a centrifuge tube, 80 ml of 4 M LiCl was added, and the mixture was stirred and then allowed to stand at 4°C for 20 hours. After centrifugation on a Hitachi RPR10 rotor at 9,000 rpm for 90 minutes, an RNA precipitate was recovered. The RNA precipitate was suspended in 50 ml of a solution comprising 4 M urea and 2 M lithium chloride and, after centrifugation on a Hitachi RPR10 rotor at 9,000 rpm for 60 minutes, the RNA was again recovered as a precipitate the RNA precipitate was dissolved in 10 ml of a solution comprising 0.1% sodium lauryl sulfate, 1 mM EDTA and 10 mM Tris-HCl (pH 7.5), and the RNA was recovered by phenol-chloroform extraction followed by ethanol precipitation. About 2.5 mg of the thus-obtained RNA was dissolved in 1 ml of a solution comprising 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. After incubation at 65°C for 5 minutes, 0.1 ml of 5 M NaCl was added. The mixture was subjected to oligo(dT)-cellulose column (P-L Biochemicals) chromatography (column volume 0.5 ml). The adsorbed, poly(A)-containing mRNA was eluted with a solution comprising 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. About 100 μg of the poly(A)-containing mRNA.

(2) cDNA synthesis and insertion of the DNA into a vector

The Okayama-Berg method [Mol. Cell. Biol., 2, 161 (1982)] was followed for cDNA synthesis and recombinant plasmid construction by insertion of the cDNA obtained. The processes therefor are outlined in Fig. 9.

To 300 µl of a solution comprising 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 10 mM NaCl, there was added 400 µg of pCDV1 [Okayama & Berg: Mol. Cell. Biol., 3, 280 (1983)] and, after further addition of 500 units of KpnI, the reaction was carried out at 37°C for 6 hours, whereby the plasmid was cleaved at the KpnI site. The DNA recovered byphenol-chloroform extraction followed by ethanol precipitation. About 200 µg of the KpnI-cleaved DNA was added to 200 µl of a solution prepared by adding dTTP in a concentration of 0.25 mM to TdT buffer and, after further addition of 81 units of TdT (P-L Biochemicals), the reaction was carried out at 37°C for 11 minutes, whereby a poly(dT) chain (about 67 dT residues) was added to each 3' end of the KpnI cleavage site of pCDV1. About 100 µg of the poly(dT) chain-tailed pCDV1 DNA was recovered from the solution by ethanol precipitation following phenol-chloroform extraction. The DNA was added to 150 µl of a solution comprising 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 100 mM NaCl and, after further addition of 360 units of EcoRI, the reaction was carried out at 37°C for 2 hours. The reaction mixture was treated by the LGT method, and a DNA fragment of about 3.1 kb was recovered. About 60 µg of the poly(dT) chain-tailed pCDV1 was thus obtained. The DNA was dissolved in 500 µl of a solution comprising 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, the solution was incubated at 65°C for 5 minutes and then cooled with ice, and 50 µl of 5 M NaCl was added. The mixture was subjected to oligo(dA)-cellulose column (Collaborative Research) chromatography. Molecules having a sufficient poly(dT) chain length were adsorbed on the column and they were eluted with a solution comprising 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA to give 27 ug of the poly(dT) chain-tailed pCDV1 (hereinafter referred to as vector primer).

Then, a linker DNA was prepared.

About 14 µg of pL1 [Okayama & Berg: Mol. Cell. Biol., 3, 280 (1983)] was added to 200 µl of a buffer comprising 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 50 mM NaCl and, after further addition of 50 units of PstI, the reaction was carried out at 37°C for 4 hours for cleavage of the pL1 DNA at the PstI site. The reaction mixture was subjected to phenol-chloroform extraction and about 13 µg of the PstI-cleaved pL1 DNA was recovered by ethanol precipitation. About 13 µg of the DNA was added to 50 µl of TdT buffer containing dGTP in a final concentration of 0.25 mM and, after further addition of 54 units of TdT (P-L Biochemicals), incubation was carried out at 37°C for 13 minutes to cause addition of a (dG) chain (about 14 dG residues) to pL1 at each PstI cleavage site 3' end. After phenol-chloroform extraction, the DNA was recovered by ethanol precipitation. The DNA was added to 100 µl of a buffer comprising 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 60 mM NaCl and, after further addition of 80 units of HindIII, incubation was carried out at 37°C for 3 hours to cause cleavage of the pL1 DNA at the HindIII site. The reaction mixture was fractionated by agarose gel electrophoresis, and a DNA fragment of about 0.5 kb was recovered by the DEAE-paper method [Dretzen et al.: Anal. Biochem., 112, 295 (1981)]. Thus was obtained the oligo(dG) chain-tailed linker DNA (hereinafter referred to simply as linker DNA).

About 2 µg of the poly(A) RNA and about 1.4 µg of the vector primer dissolved in 22.3 µl of a solution comprising 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 30 mM KCl, 0.3 mM DTT, 2 mM dNTP (dATP, dTTP, dGTP and dCTP) and 10 units of ribonuclease inhibitor (P-L Biochemicals), 10 units of reverse transcriptase (Seikagaku Kogyo) was added, and incubation was carried out at 41°C for 90 minutes to cause the mRNA to synthesize a DNA complementary thereto. The reaction mixture was subjected to phenol-chloroform extraction followed by ethanol precipitation, whereby the vector primer DNA with the RNA-DNA double strand added thereto was recovered. The DNA was dissolved in 20 µl of TdT buffer containing 66 mM dCTP and 0.2 µg of poly(A), 14 units of TdT (P-L Biochemicals) was added, and incubation was performedat 37°C for 2 minutes to cause addition of a (dC) chain (20 dC residues) to the 3' end of the cDNA. The reaction mixture was subjected to phenol-chloroform extraction and then the (dC) chain-tailed cDNA-vector primer DNA was recovered by ethanol precipitation. The DNA was dissolved in 400 µl of a solution comprising 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 60 mM NaCl, 20 units of HindIII was added, and incubation was carried out at 37°C for 2 hours for cleavage at the HindIII site. Phenol-chloroform extraction of the reaction mixture and ethanol precipitation gave 0.5 picomole of the (dC) chain-tailed cDNA-vector primer DNA. The DNA (0.2 picomole) was dissolved in 100 µl of a solution comprising 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl and 1 mM EDTA, and incubation was carried out at 65°C, 42°C and 0°C for 10 minutes, 25 minutes and 30 minutes, respectively, in that order. A total volume of 100 µl of a reaction medium was prepared according to the following composition: 20 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1 M KCl and 0.1 mM β-NAD. To this reaction medium, there was added 25 units of E.

coli DNA ligase (New England Bio-Labs), and incubation was performed at 11°C for 18 hours. The reaction medium was supplemented with 40 µM each dNTP and with β-NAD in a final concentration of 0.15 mM and, after addition of 10 units of E. coli DNA ligase, 20 units of E . coli DNA polymerase I (P-L Biochemicals) and 10 units of E. coli ribonuclease H (P-L Biochemicals), incubation was carried out at 12°C for 1 hour and then at 25°C for 1 hour. The above reaction procedure caused circularization of the cDNA-containing recombinant DNA and substitution of the RNA portion of the RNA-DNA double strand by the corresponding DNA. Thus, the recombinant plasmid was formed in the form of a completely doub-stranded DNA.

(3) Selection of the human LT cDNA-containing recombinant DNA

The recombinant plasmid obtained as described in (2) was used to transform E. coli C600SF8- [Cameron: Proc. Natl. Acad. Sci. USA, 72, 3416 (1975)] by the method of Scott et al. [Katsuya Shigesada: Saibo Kogaku (Cell Technology), 2, 616 (1983)]. About 30,000 colonies obtained were fixed on a nitrocellulose filter. One strain capable of strongly associating, at 52°C, with a probe prepared by labeling, with ^{32}P, the 17-base synthetic DNA 5'-GATCCCCGGCCTGCCTG-3' corresponding to the base sequence of part of the 5' nontranslational region of the human LT cDNA isolated by Genentech [Patrick W. Gray et al.: Nasture, 312, 721 (1984)] was selected [Grunstein-Hogness method: Proc. Natl. Acad. Sci. USA, 72, 3961 (1975)]. The whole base sequence of the cDNA of the plasmid pLT1 carried by this strain was determined by the dideoxy sequencing method using M13 phage. As a result, it was found that the pLT1 DNA codes for human LT.

(4) Construction of the recombinant plasmid pLA1

In a total of 50 µl of a solution (hereinafter referred to as "Y-0 buffer") containing 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$ and 6 mM 2-mercaptoethanol, there was dissolved 5 ug of pLT1 (4.7 kb) obtained by the procedure described in the preceding section, 10 units of the restriction enzyme XhoII (Boehringer Mannheim) was added, and the cleavage reaction was conducted at 37°C for 2 hours. Then, NaCl was added in a final concentration of 150 mM, 10 units of the restriction enzyme NsiI (New England Bio-Labs) was added, and the cleavage reaction was carried out at 37°C for further 3 hours. From the reaction mixture, there was obtained, by the LGT method, about 0.3 µg of an about 750 bp DNA fragment (XhoII-NsiI fragment) containing most of the human LT DNA.

Separately, 20 µg of pLT1 was dissolved in 200 µl of Y-50 buffer, 40 units of the restriction enzyme HaeIII was added, and the cleavage reaction was performed at 37°C for 2 hours. Then, NaCl was added in a final concentration of 150 mM, 40 units of NsiI was added, and the cleavage reaction was carried out at 37°C for further 3 hours. Polyacrylamide gel electrophoresis of the reaction mixture gave about 40 ng of an about 50 bp DNA fragment (Hae III-NsiI fragment) containing the N-terminal portion of human LT.

Further, separately, 3 µg of pGEL1 (3.4 kb) was dissolved in a total of 30 µl of Y-100 buffer, 6 units each of the restriction enzymes StuI and BglII were added, and the cleavage reaction was carried out at 37°C for 3 hours.

From the reaction mixture, there was obtained, by the LGT method, about 1.0 µg of an Ap^r gene-containing DNA fragment of about 2.3 kb (Stu I-BglII fragment).

Then, 0.2 µg of the pLT1-derived XhoII-NsiI fragment (about 750 bp), 20 ng of the pLT1-derived HaeIII-Nsi I fragment (about 50 bp) and 0.6 µg of the pGEL1-derived Stu I-BglII fragment (about 2.3 kb) were dissolved in a total of 20 µl of T4 ligase buffer, 2 units of T4 DNA ligase (Takara Shuzo) was further added to this mixture solution, and the reaction was carried out at 4°C for 18 hours.

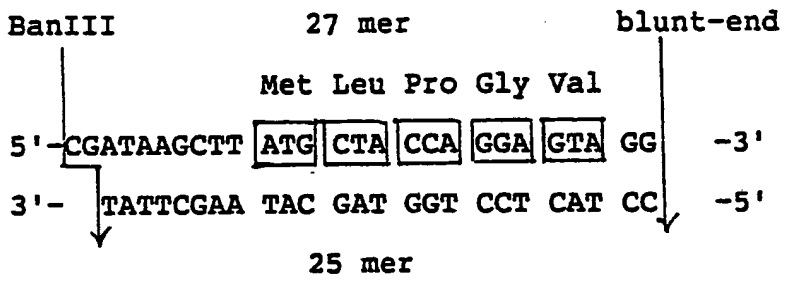
The recombinant plasmid DNA thus obtained was used to transform E. coli KM430 by the method of Cohen et al., and an Ap^r colony was obtained. The plasmid DNA was isolated and purified from this transformant by a known method, and the structure of the plasmid was analyzed by cleavage of said plasmid DNA with restriction enzymes such as StuI. As a result, it was confirmed that the desired plasmid had been obtained. This recombinant plasmid is named pLA1.

(5) Construction of the LT expression plasmid pLSA1

An E. coli KM430 transformant harboring pLA1 (3.1 kb) obtained as described in the preceding section was cultured, and the pLA1 DNA was prepared from cultured cells thereof in the conventional manner. In 30 µl of Y-100 buffer, there was dissolved 3 µg of the pLA1 DNA obtained, 3 units each of StuI and BglII were added, and the cleavage reaction was conducted at 37°C for 3 hours. From the reaction mixture, there was obtained, by the LGT method, about 0.5 µg of an about 790 bp DNA fragment (StuI-BglII fragmen6t) containing most of the human LT gene.

Separately, 3 µg of pKYL10 prepared by the method described in US Patent 4,686,191 was dissolved in 30 µl of Y-100 buffer, 6 units each of the restriction enzymes BanIII and PstI were added, and the cleavage reaction was carried out at 37°C for 3 hours. From the reaction mixture, there was obtained, by the LGT method, a tryptophan promoter (Ptrp)-containing DNA fragment of about 1.1 kb (BanIII-PstI fragment). Further, 2 µg of pGEL1 (3.4 kb) was dissolved in 20 µl of Y-100 buffer, 4 units each of therestriction enzymnes HindIII, BamHI and PstI were added, and the cleavage reaction was carried out at 37°C for 3 hours. From the reaction mixture, there was obtained, by the LGT method, about 0.7 µg of a lipoprotein-derived terminator-containing DNA fragment of about 1.7 kb (PstI-BamHI fragment).

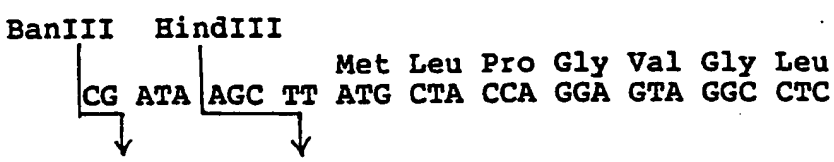
Separately, for such reasons as the necessity of providing the sequence from the N terminus of the mature human LT polypeptide, namely Leu (CTA) to the second base (GG) of the 5th amino acid Gly (GGC) as well as the initiation codon (ATG) required for expression and the necessity of adjusting the distance between the SD sequence downstream from Ptrp and ATG to an appropriate length of 6-18 bp, the following DNA linker was synthesized:



First, the 27-mer and 25-mer single-strand DNAs were synthesized by the ordinary phosphotriester method. The 27-mer and 25-mer (each 20 picomoles) were dissolved in a totalof 40 µl of T4 kinase buffer, 6 units of T4 polynucleotide kinase (Takara Shuzo) was added, and the phosphorylation reaction was carried out at 37°C for 60 minutes.

Then, 0.3 µg of the pLA1-derived StuI-BglII fragment (about 790 bp), 0.4 µg of the BanIII-PstI fragment (about 1.1 kb) of the expression vector pKYP10 and 0.6 pg of the pGEL1-derived PstI-BamHI fragment (about 1.7 kb), each obtained as described above, were dissolved in 25 µl of T4 ligase buffer, and about 1 picomole of the above DNA linker was added to this mixture solution. After further addition of 6 units of T4 DNA ligase to this mixture, the ligation reaction was conducted at 4°C for 18 hours.

The recombinant plasmid-containing reaction mixture was used to transform E. coli KM430, and an Ap'colony was obtained. The plasmid DNA was recovered from cultured cells of this colony. The structure of the plasmid obtained was confirmed by cleavage with the restriction enzymes EcoRI, BanIII, PstI, Hin dIII and BglII followed by agarose gel electrophoresis. This plasmid is named pLSA1. The base sequence of pLSA1 in the vicinity of BanIII and HindIII was confirmed by the Maxam-Gilbert method [(A. M. Maxam et al.: Proc. Natl. Acad. Sci. USA, 74, 560 (1977)] to be as follows:



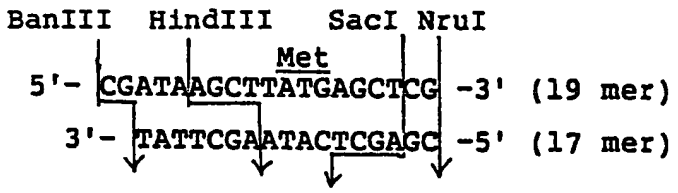
Reference Example 4

Construction of the ATG vector pTrS20

By following the procedure shown in Fig. 13, the ATG vector pTrS20 in which the distance between the SD sequence and the initiation codon ATG is 14 bases and which has an SacI site immediately behind the ATG codon was constructed.

First, 3 µg of pKYP10 prepared by the method described in US Patent 4,686,191 was dissolved in 30 µl of Y-100 buffer, 6 units each of the restriction enzymes Ban III and NruI (New England Bio-Labs) were added, and the cleavage reaction was conducted at 37°C for 3 hours. From the reaction mixture, there was obtained, by the LGT method, about 0.5 µg of a Ptrp-containing DNA fragment of about 3.8 kb (BanIII-Nru I fragment).

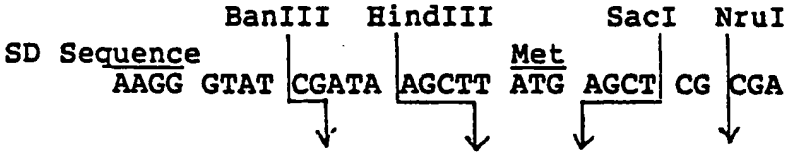
Separately, the following DNA linker was synthesized by the phosphotriester method for providing the initiation codon ATG downstream from Ptrp:



The 19-mer and 17-mer synthetic DNAs (each 10 picomoles) were dissolved in a total of 20 µl of a solution containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM EDTAs and 1 mM ATP, 3 units of T4 polynucleotide kinase (Takara Shuzo) was added, and the phosphoryolation reaction was effected at 37°C for 60 minutes.

Then, 0.1 µg of the pKYP10-derived BanIII-NruI fragment (about 3.8 kb) obtained as described above and about 0.5 picomole of the above DNA linker were dissolved in 20 µl of T4 ligase buffer, 2 units of T4 DNA ligase was further added, and the ligation reaction was carried out at 4°C for 18 hours.

The recombinant plasmid mixture obtained was used to transform E. coli HB101 [Boliver et al.: Gene, 2, 75 (1977)], and an Ap^r colony was obtained. From cultured cells of this colony, there was recovered the plasmid DNA . The structure of the plasmid obtained was confirmed by cleavage with the restriction enzymes EcoRI, BanIII, Hin dIII, SacI and NruI, followed by agarose gel electrophoresis. This plasmid was named pTrS20 (Fig. 13). The base sequence of pTrS20 in the neighborhood of the BanIII and Hin dIII sites was confirmed by the dideoxy sequencing method using M13 phage to be as follows:



Claims

1. A novel polypeptide having an amino acid sequence as derived from the amino acid sequence of the human granulocyte colony stimulating factor (hG-CSF) polypeptide by substitution of at least one amino acid by an amino acid different therefrom.

2. The novel polypeptide according to Claim 1, wherein at least one amino acid on the N terminus side thereof differs from the amino acid in the corresponding position of the hG-CSF polypeptide.

3. The novel polypeptide according to Claim 1, wherein at least one amino acid out of the Ist to 17th amino acids on the N terminus side thereof differs from the amino acid in the corresponding position of the hG-CSF polypeptide.

4. The novel polypeptide according to claim 1, wherein at least one amino acid out of the 1st to 6th amino acids on the N terminus side and the 17th amino acid thereof differs from the amino acid in the corresponding position of the hG-CSF polypeptide.

5. A DNA coding for a polypeptide having an amino acid sequence as derived from the amino acid sequence of the hG-CSF polypeptide by substitution of at least one amino acid by an amino acid different therefrom.

6. A recombinant plasmid comprising a plasmid DNA and, as an insert therein, a DNA fragment coding for a polypeptide having an amino acid sequence as derived from the amino acid sequence, of the hG-CSF polypeptide by substitution of at least one amino acid by an amino acid different therefrom.

7. The recombinant plasmid according to Claim 6, wherein the plasmid DNA is a tryptophan promoter-containing plasmid DNA, with said DNA fragment inserted in said plasmid DNA at a site downstream from the tryptophan promoter.

8. The recombinant plasmid according to Claim 6 or 7, wherein said DNA fragment has a sequence such that at least one base out of the 1st to 51st bases of the base sequence has been substituted by a different base.

9. The recombinant plasmid according to Claim 6, 7 or 8 which is selected from the group consisting of pCfTL38, pCfTL41, pCfTL23, pCfTL35, pCfBB101, pCfBC42BI, pCfBC45, pCfBC52, pCfBC59, pCfBC76, pCfBC77, pCfBC93, pCfBC95, pCfBC97, pCfBD28, pCfBD56, pCfBD82, pCfTM14, pCfTM17, pCfTM113, pCfTAArg4S, pCfTAArg4, pCfBD28A17and pCfBD28T17.

10. A microorganism harboring a recombinant plasmid comprising a plasmid DNA and, as an insert therein, a DNA fragment coding for a polypeptide having an amino acid sequence as derived from the amino acid sequence of the hG-CSF polypeptide by substitution of at least one amino acid by an amino acid different therefrom.

11. The microorganism according to Claim 10, wherein said microorganism belongs to the species E-scherichia coli.

12. The microorganism according to Claim 10, wherein said recombinant plasmid is selected from the group consisting of pCfTL38, pCfTL41, pCfTL23, pCfTL35, pCfBB101, pCfBC42B1, pCfBC45, pCfBC52, pCfBC59, pCfBC76, pCfBC77, pCfBC93, pCfBC95, pCfBC97, pCfBD28, pCfBD56, pCfBD82, pCfTM14, pCfTM17, pCfTM113, pCfTAArg4S, pCfTAArg4, pCfBD28A17 and pCfBD28T17.

13. A method of producing a polypeptide having an amino acid sequence derived from the amino acid sequence of the hG-CSF polypeptide by substitution of at least one amino acid by an amino acid different therefrom, which method comprises (a) cultivating in a medium a microorganism harboring a recombinant plasmid comprising a plasmid DNA and, as an insert therein, a DNA fragment coding for said polypeptide to thereby cause formation and accumulation of said polypeptide in the culture, and (b) recovering said polypeptide from said culture.

14. The method according to Claim 13 , wherein said microorganism belongs to the species E-scherichia coli.

15. The method according to Claim 13, wherein said recombinant plasmid is selected from the group consisting of pCfTL38, pCfTL41, pCfTL23, pCfBB101, pCfBC42B1, pCfBC45, pCfBC52, pCfBC59, pCfBC76, pCfBC77, pCfBC93, pCfBC95, pCfBC97, pCfBD28, pCfBD56, pCfBD82, pCfTM14, pCfTM17, pCfTM113, pCfTAArg4S, pCfTAArg4, pCfBD28A17 and PCfBD28T17.

16. A polypeptide derived from the hG-CSF polypeptide by deletion of at least one amino acid.

17. The polypeptide according to Claim 16, wherein at least one amino acid out of the 1st to 17th amino acids on the N terminus portion of the hG-CSF polypeptide is missing.

18. The polypeptide according to Claim 17, wherein a peptide in the 1st to 4th, 1st to 5th, 1st to 6th, 1st to 7th or 1st to 11th amino acids on the N terminus portion of the hG-CSF polypeptide is missing.

19. A DNA coding for a polypeptide as derived from the hG-CSF polypeptide by deletion of at least one amino acid.

20. A DNA coding for a polypeptide as derived from the hG-CSF polypeptide by deletion of at least one amino acid out of the 1st to 11th amino acids on the N terminus portion thereof.

21. The DNA according to Claim 20 , wherein, in the polypeptide encoded by it, a peptide comprising the 1st to 4th, 1st to 5th, 1st to 6 th, 1st to 7th or 1st to 11th amino acids on the N terminus portion of the hG-CSF polypeptide is missing.

22. A recombinant plasmid which comprises a plasmid DNA and, as an insert therein, a DNA fragment coding for a polypeptide as derived from the hG-CSF polypeptide by deletion of at least one amino acid.

23. The recombinant plasmid according to Claim 22 which comprises a plasmid DNA and, as an insert therein, a DNA fragment coding for a polypeptide as derived from the hG-CSF polypeptide by deletion of at least one amino acid out of the 1st to 11th amino acids on the N terminus portion thereof.

24. The recombinant plasmid according to Claim 23 which comprises a plasmid DNA and, as an insert therein, a DNA fragment coding for a polypeptide as derived from the hG-CSF polypeptide by deletion of a peptide comprising the 1st to 11th amino acids on the N terminus portion thereof.

25. The recombinant plasmid according to Claim 24, wherein a tryptophan promoter-containing plasmid DNA is used as said plasmid DNA, with said DNA fragment inserted in the plasmid DNA at a site downstream from the tryptophan promoter..

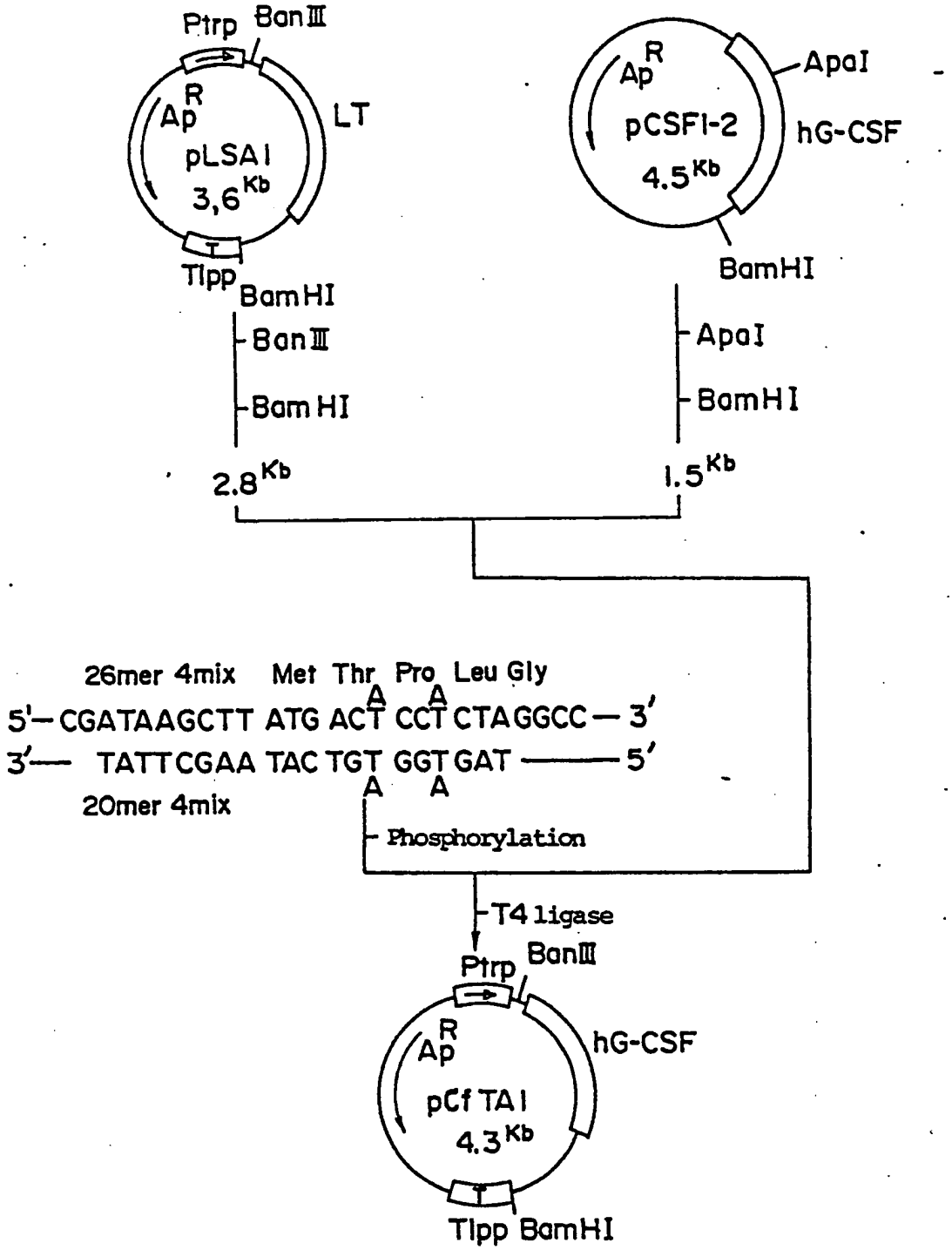
26. The recombinant plasmid according to Claim 25 which is selected from the group consisting of pCfTNS7, pCfTNS301, pCfTNS401 and pCfTNS501.

27. The polypeptide according to Claim 16, wherein, in addition to amino acid deletion, the 17th amino acid cystein (Cys) of the hG-CSF polypeptide has been replaced by serine (Ser).

28. A method of producing a polypeptide having an amino acid sequence as derived from the amino acid sequence of the hG-CSF polypeptide by deletion of at least one amino acid, which method comprises cultivating in a medium a microorganism harboring a recombinant plasmid comprising a plasmid DNA and, as an insert therein, a DNA fragment coding for said polypeptide to thereby cause for mation and accumulation of said polypeptide in the culture, and recovering said polypeptide from said culture.

29. A method of producing a novel polypeptide having an amino acid sequence as derived from the amino acid sequence of the hG-CSF polypeptide by substitution of the 17th amino acid (cysteine) by serine and deletion of 4-7 amino acids on the N terminus side thereof, which method comprises subjecting a polypeptide as derived from the hG-CSF polypeptide by substitution of the 17th amino acid (cysteine) by serine and of at least one amino acid out of the 1st to 6th amino acids on the N terminus side thereof by an amino acid different therefrom to the action of protease in an aqueous medium to thereby cause formation of said novel polypeptide in the reaction mixture, and recovering said novel polypeptide from said reaction mixture.

Fig. 1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Ap R
LT
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pCSF1-2
4.5 Kb
hG-CSF
BamHI
ApaI
BamHI
1.5 Kb
26mer 4mix
Met Thr Pro Leu Gly
5'— CGATAAGCTT ATG ACT CCT CTAGGCC — 3'
3'— TATTCGAA TAC TGT GGT GAT ——— 5'
A A
A A
20mer 4mix
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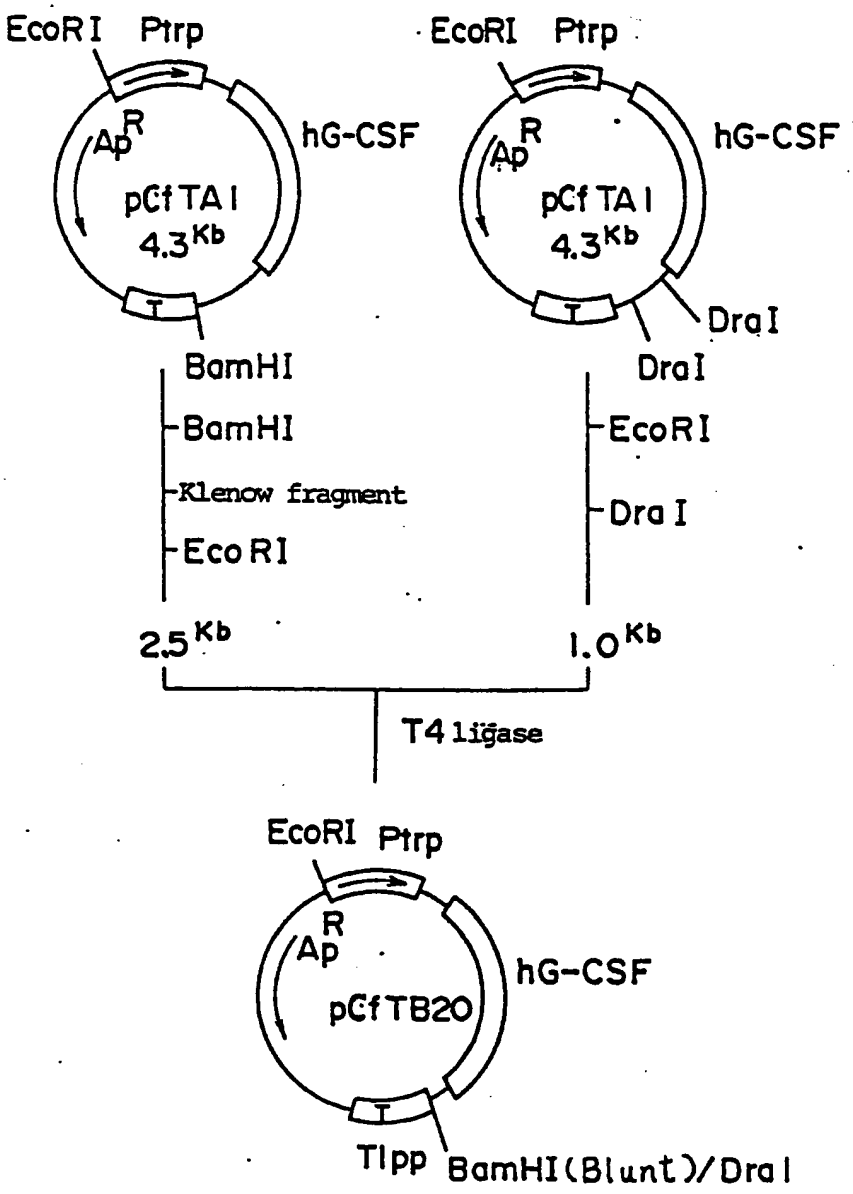
Tlpp
BamHI

Fig. 2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Ptrp
hG-CSF
Ap R
pCfTA1
4.3 Kb
T
BamHI
BamHI
Klenow fragment
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2.5 Kb
EcoRI
Ptrp
hG-CSF
Ap R
pCfTA1
4.3 Kb
T
DraI
DraI
EcoRI
DraI
1.0 Kb
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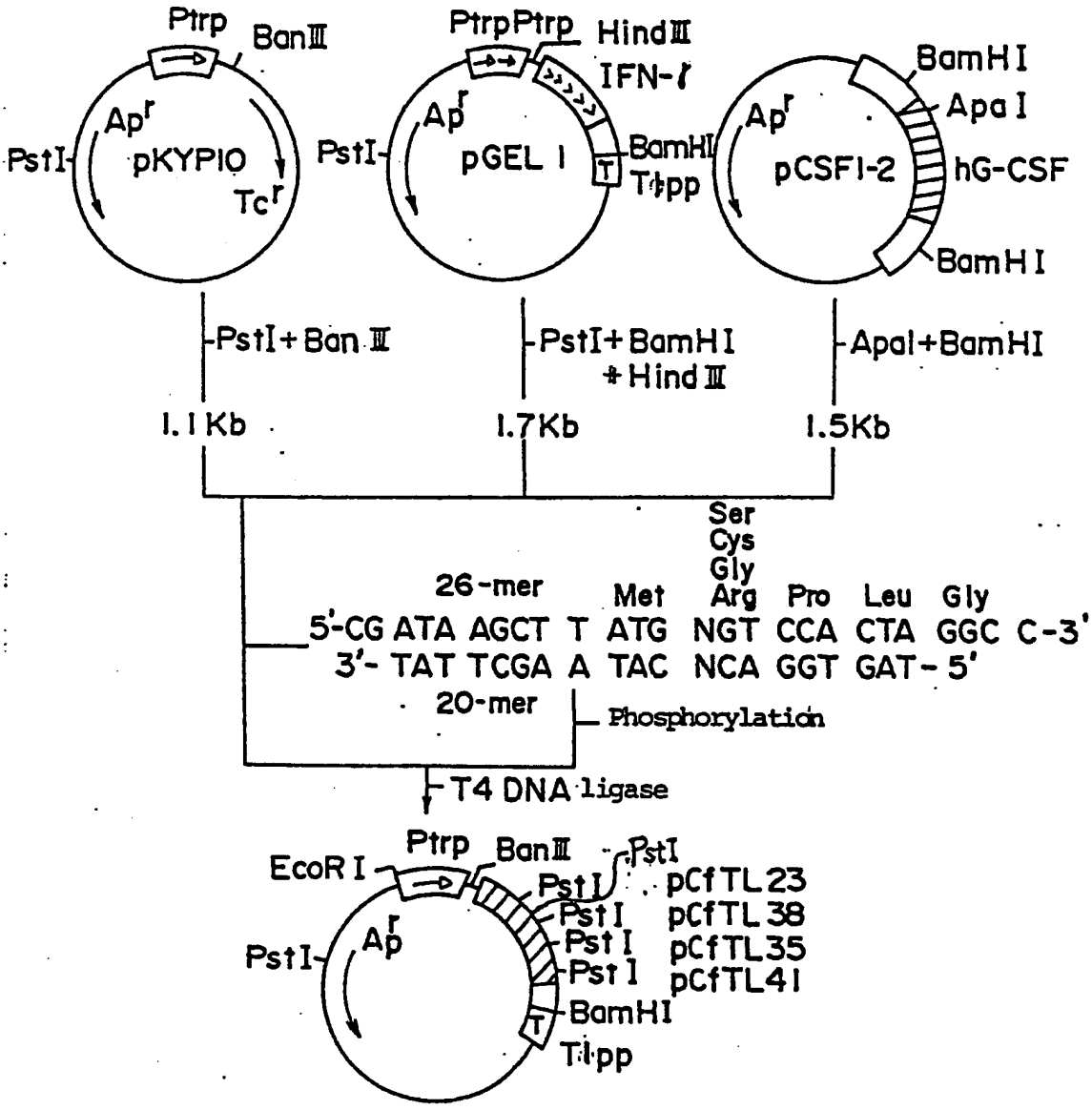
Tlpp
BamHI(Blunt)/DraI

Fig. 3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BamHI
ApaI
pCSF1-2
hG-CSF
BamHI
PstI+BanIII
PstI+BamHI
+HindIII
ApaI+BamHI
1.1Kb
1.7Kb
1.5Kb
Ser
Cys
Gly
26-mer
Met
Arg
Pro
Leu
Gly
5'-CG ATA AGCT T ATG NGT CCA CTA GGC C-3'
3'- TAT TCGA A TAC NCA GGT GAT- 5'
20-mer
Phosphorylation
T4 DNA ligase
Ptrp
BanIII
PstI
EcoR I
PstI
pCfTL23
PstI
pCfTL38
PstI
pCfTL35
PstI
pCfTL41
PstI
Ap^r
BamHI
T
Tιpp

Fig. 4

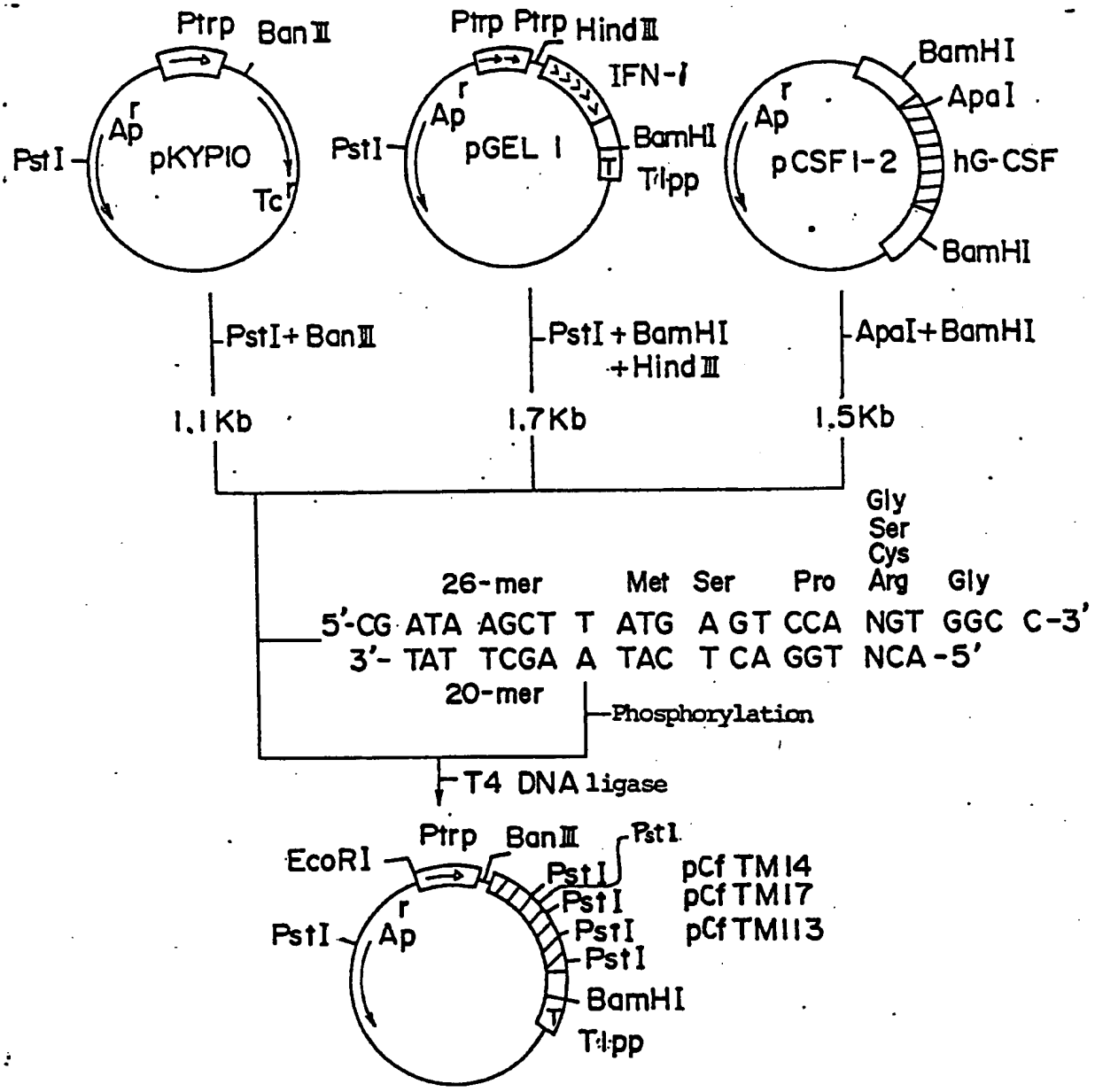

Ptrp
BanⅢ
PstI
Ap
pKYP10
Tc
Ptrp Ptrp
HindⅢ
IFN-I
pGEL 1
BamHI
T
T1pp
BamHI
ApaI
pCSF1-2
hG-CSF
BamHI
PstI+BanⅢ
PstI+BamHI
+HindⅢ
ApaI+BamHI
1.1Kb
1.7Kb
1.5Kb
Gly
Ser
Cys
26-mer
Met Ser Pro Arg Gly
5'-CG ATA AGCT T ATG A GT CCA NGT GGC C-3'
3'- TAT TCGA A TAC T CA GGT NCA -5'
20-mer
Phosphorylation
T4 DNA ligase
EcoRI
Ptrp
BanⅢ
PstI
PstI
PstI
PstI
PstI
pCf TM14
pCf TM17
pCf TM113
BamHI
T1pp

Fig. 5

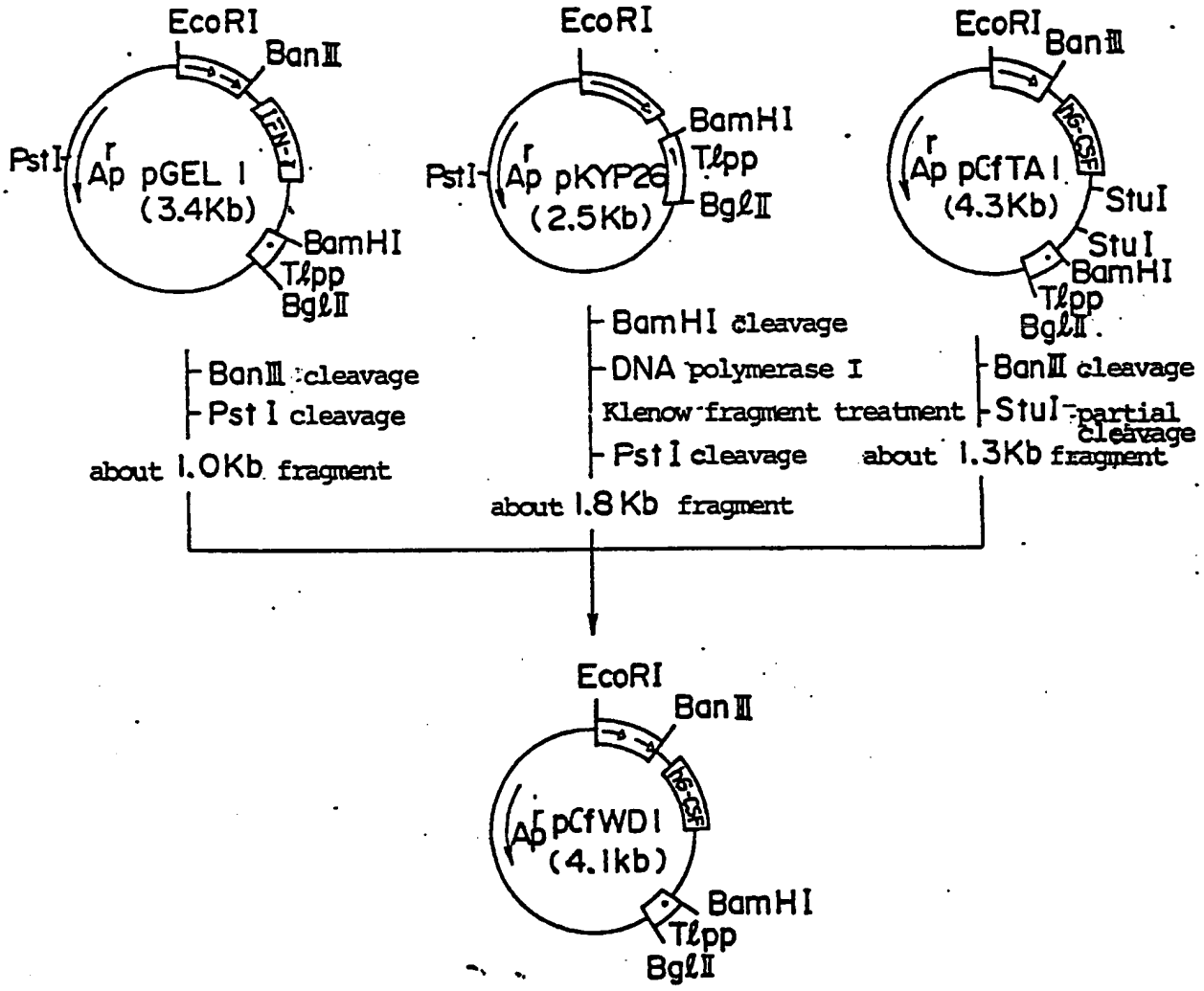

EcoRI
BanⅢ
PstI
Ap r
pGEL 1
(3.4Kb)
hEN-1
BamHI
Tℓpp
BgℓⅡ
EcoRI
BamHI
PstI
Ap r
pKYP26
(2.5Kb)
Tℓpp
BgℓⅡ
EcoRI
BanⅢ
Ap r
pCfTA1
(4.3Kb)
hG-CSF
StuI
StuI
BamHI
Tℓpp
BgℓⅡ
BamHI cleavage
DNA polymerase I
Klenow fragment treatment
PstI cleavage
about 1.8 Kb fragment
BanⅢ cleavage
PstI cleavage
about 1.0Kb fragment
BanⅢ cleavage
StuI partial cleavage
about 1.3Kb fragment
EcoRI
BanⅢ
Ap r
pCfWD1
(4.1kb)
hG-CSF
BamHI
Tℓpp
BgℓⅡ

Fig. 6

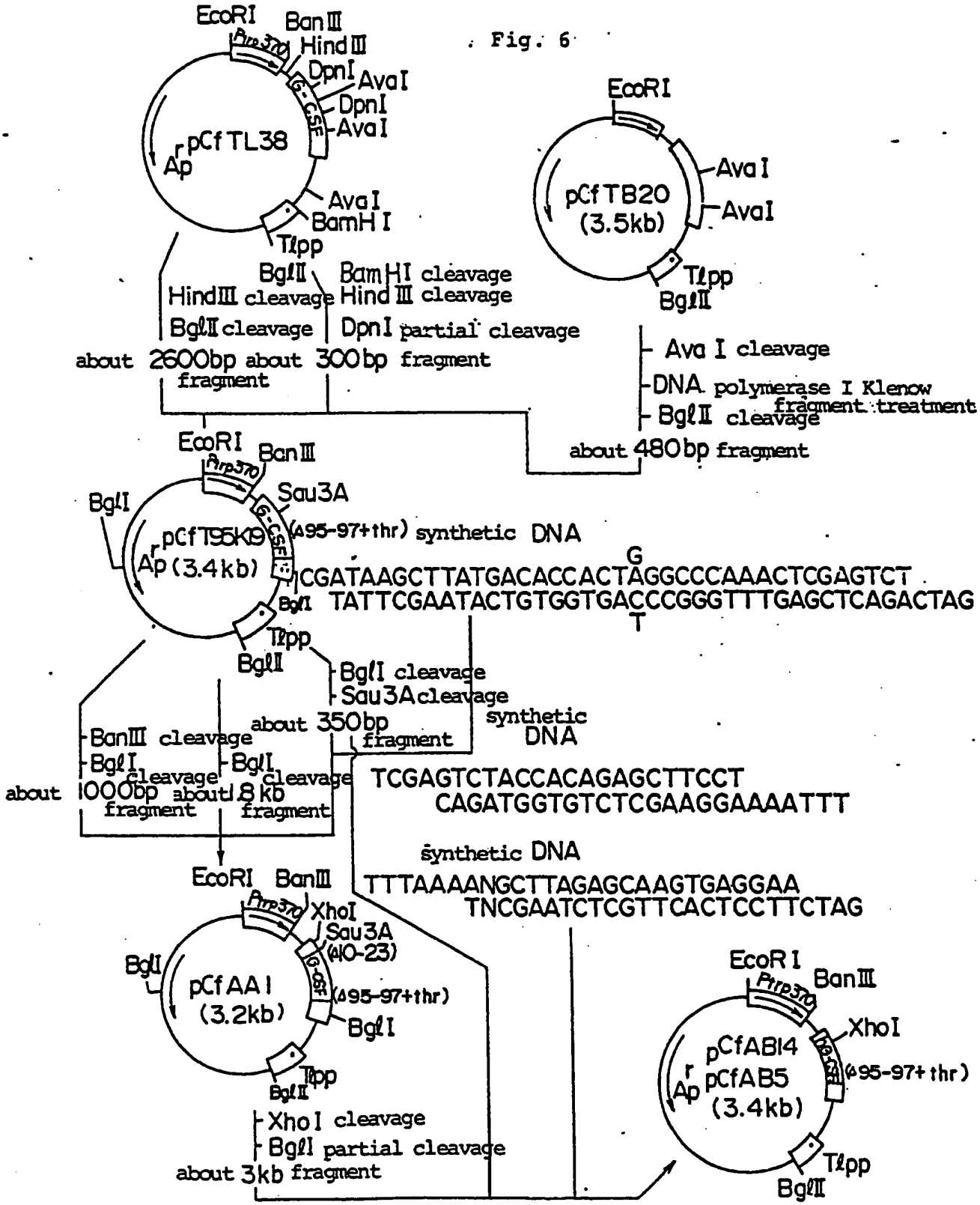

EcoRI
BanIII
Hind III
DpnI
AvaI
DpnI
AvaI
Ptrp370
G-CSF
pCfTL38
Ap
AvaI
BamHI
Tlpp
EcoRI
AvaI
AvaI
pCfTB20
(3.5kb)
Tlpp
BglII
BglII
BamHI cleavage
Hind III cleavage
Hind III cleavage
BglII cleavage
DpnI partial cleavage
about 2600bp fragment
about 300bp fragment
Ava I cleavage
DNA polymerase I Klenow fragment treatment
BglII cleavage
about 480bp fragment
EcoRI
BanIII
Ptrp370
Sau3A
BglI
pCfT95K9
Ap (3.4kb)
G-CSF
(Δ95-97+thr) synthetic DNA
CGATAAGCTTATGACACCACTAGGCCCAAACTCGAGTCT
TATTCGAATACTGTGGTGACCCGGGTTTGAGCTCAGACTAG
G
T
BglI
Tlpp
BglII
BglI cleavage
Sau3A cleavage
about 350bp fragment
synthetic DNA
BanIII cleavage
BglI cleavage
BglI cleavage
about 1000bp fragment
about 1.8kb fragment
TCGAGTCTACCACAGAGCTTCCT
CAGATGGTGTCTCGAAGGAAAATTT
synthetic DNA
TTTAAAANGCTTAGAGCAAGTGAGGAA
TNCGAATCTCGTTCACTCCTTCTAG
EcoRI
BanIII
Ptrp370
XhoI
Sau3A
(Δ10-23)
BglI
pCfAA1
(3.2kb)
G-CSF
(Δ95-97+thr)
BglI
Tlpp
BglII
EcoRI
BanIII
Ptrp370
XhoI
pCfAB14
pCfAB5
(3.4kb)
Ap
G-CSF
(Δ95-97+thr)
Tlpp
BglII
XhoI cleavage
BglI partial cleavage
about 3kb fragment

Fig. 7

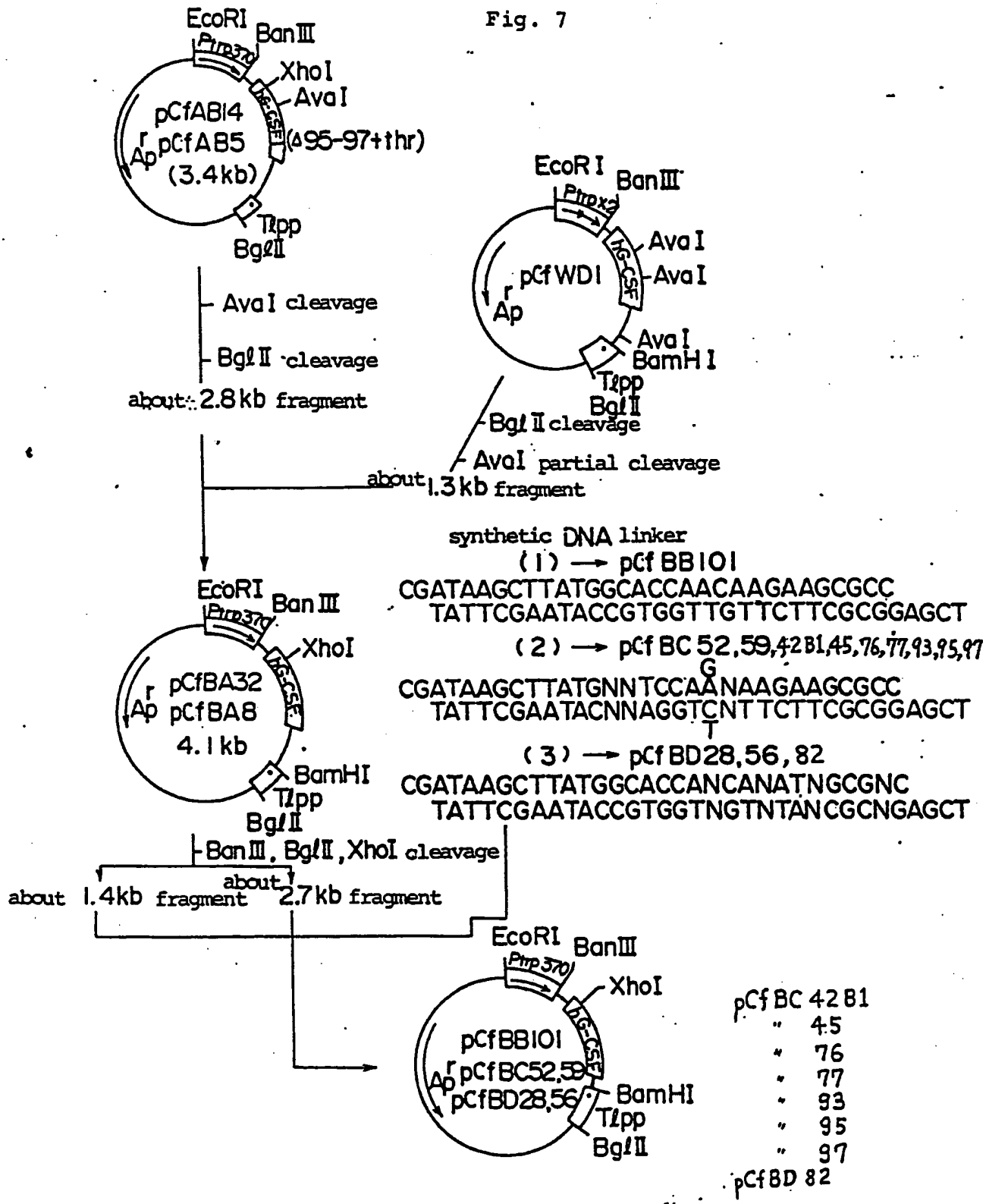

EcoRI
BanIII
Ptrp370
XhoI
AvaI
hG-CSF
pCfAB14
pCfAB5
(Δ95-97+thr)
(3.4kb)
Ap
TIpp
BglII
AvaI cleavage
BglII cleavage
about 2.8kb fragment
EcoRI
BanIII
Ptrp370
AvaI
AvaI
pCfWD1
Ap
AvaI
BamHI
TIpp
BglII
BglII cleavage
AvaI partial cleavage
about 1.3kb fragment
synthetic DNA linker
(1) → pCfBB101
CGATAAGCTTATGGCACCAACAAGAAGCGCC
TATTCGAATACCGTGGTTGTTCTTCGCGGAGCT
(2) → pCfBC52,59,42B1,45,76,77,93,95,97
G
CGATAAGCTTATGNNTCCAANAAGAAGCGCC
TATTCGAATACNNAGGTCNTTCTTCGCGGAGCT
T
(3) → pCfBD28,56,82
CGATAAGCTTATGGCACCANCANATNGCGNC
TATTCGAATACCGTGGTNGTNTANCGCNGAGCT
EcoRI
BanIII
Ptrp370
XhoI
hG-CSF
pCfBA32
pCfBA8
4.1kb
Ap
BamHI
TIpp
BglII
BanIII, BglII, XhoI cleavage
about 1.4kb fragment
about 2.7kb fragment
EcoRI
BanIII
Ptrp370
XhoI
hG-CSF
pCfBB101
pCfBC52,59
pCfBD28,56
Ap
BamHI
TIpp
BglII
pCfBC 42 B1
" 45
" 76
" 77
" 93
" 95
" 97
pCfBD 82

Fig. 8

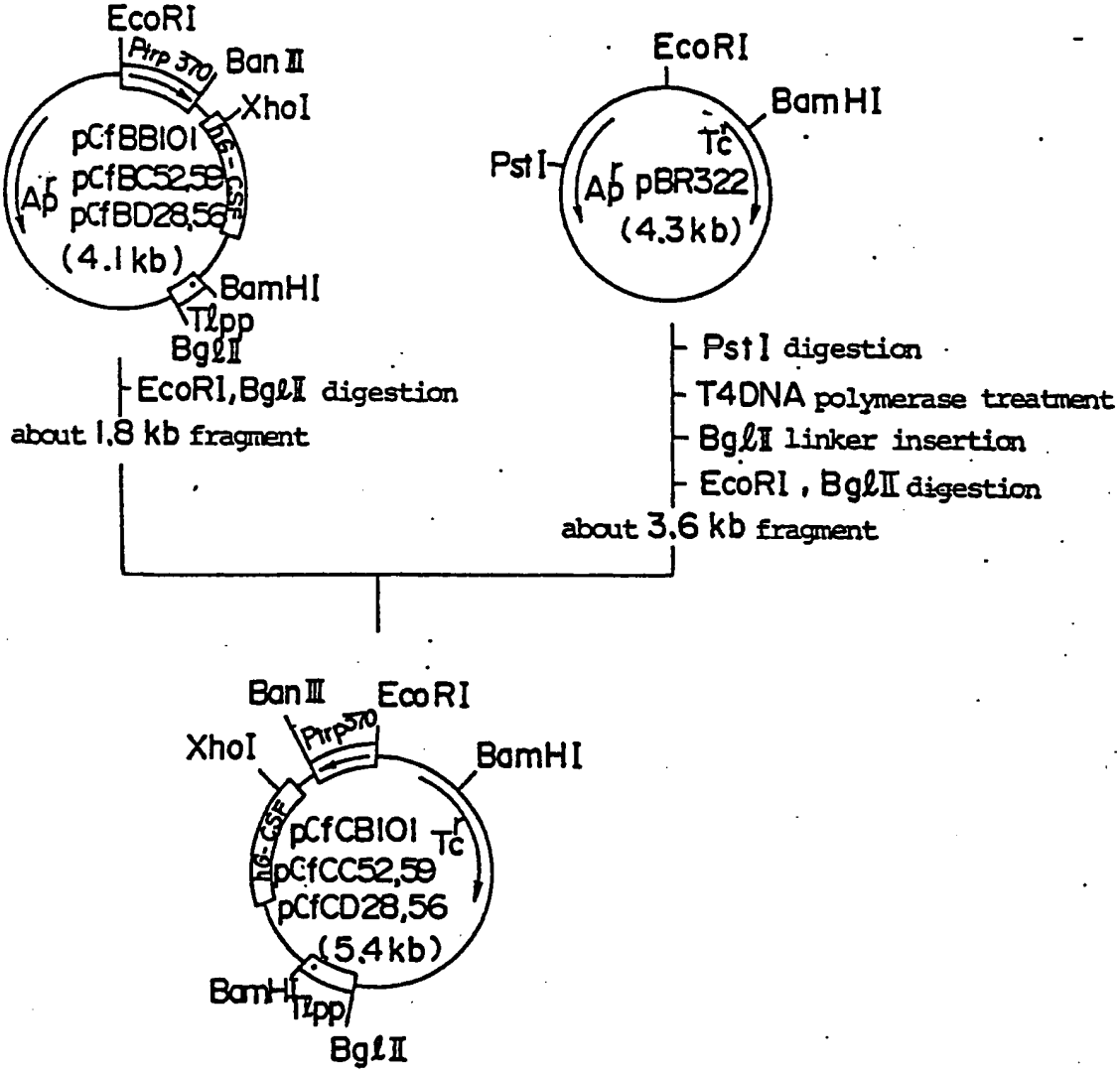

EcoRI
Ptrp 370
Ban II
XhoI
pCfBB101
pCfBC52,59
pCfBD28,56
(4.1 kb)
Ap r
hG-CSF
BamHI
Tlpp
BglII
EcoRI, BglI digestion
about 1.8 kb fragment
EcoRI
BamHI
Tc r
PstI
Ap r pBR322
(4.3 kb)
PstI digestion
T4DNA polymerase treatment
BglII linker insertion
EcoRI , BglII digestion
about 3.6 kb fragment
Ban II
EcoRI
Ptrp370
XhoI
BamHI
hG-CSF
pCfCB101 Tc r
pCfCC52,59
pCfCD28,56
(5.4 kb)
BamHI
Tlpp
BglII

Fig. 9 (1)

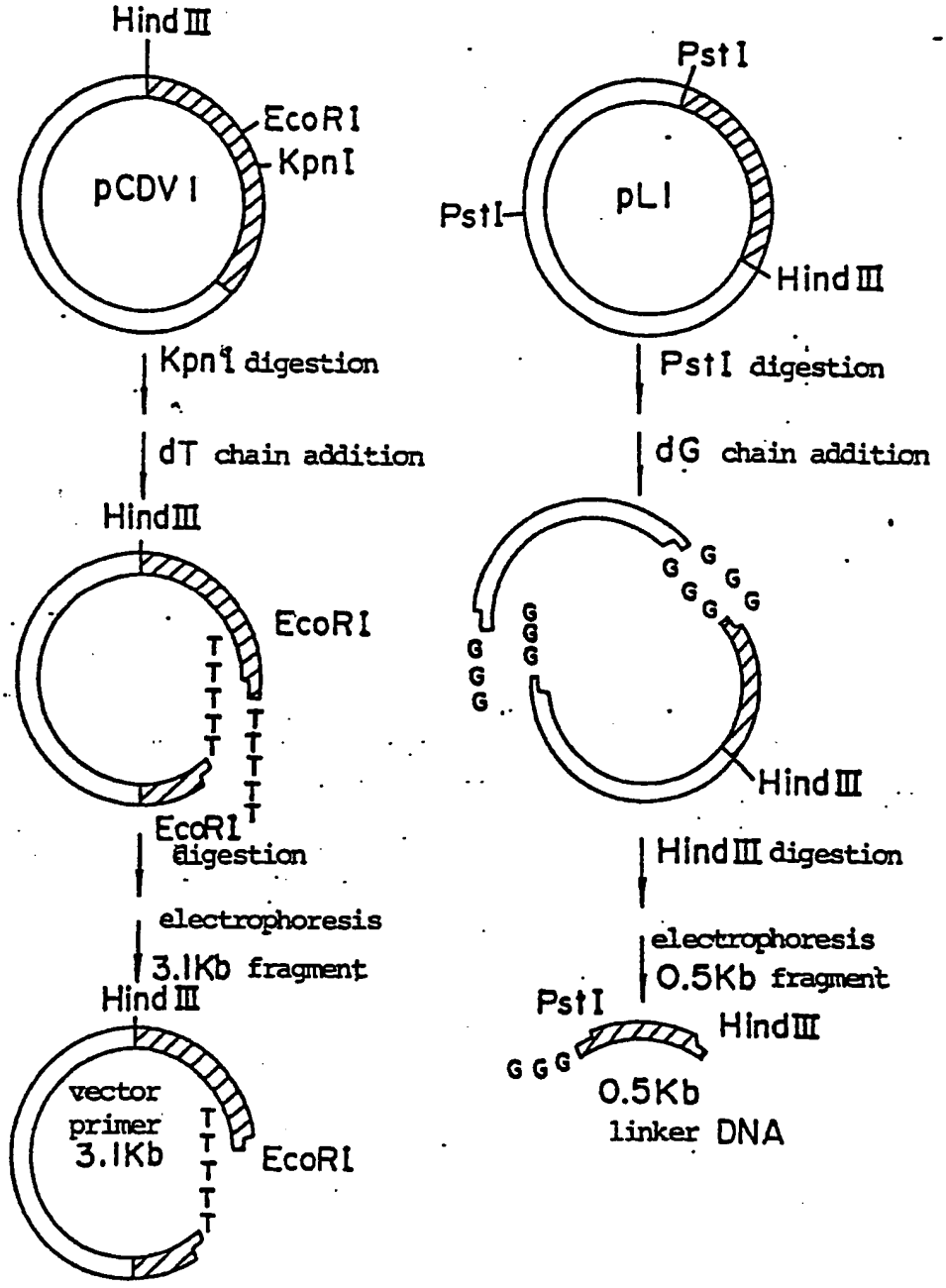

Hind III
EcoR I
Kpn I
pCDV I
Kpn I digestion
dT chain addition
Hind III
EcoR I
T T T T T T T T T T
EcoR I digestion
electrophoresis
3.1Kb fragment
Hind III
vector primer 3.1Kb
EcoR I
Pst I
pL I
Pst I
Hind III
Pst I digestion
dG chain addition
G G G G G G G G G G
Hind III
Hind III digestion
electrophoresis
0.5Kb fragment
Pst I
Hind III
G G G
0.5Kb linker DNA

Fig. 9 (2)

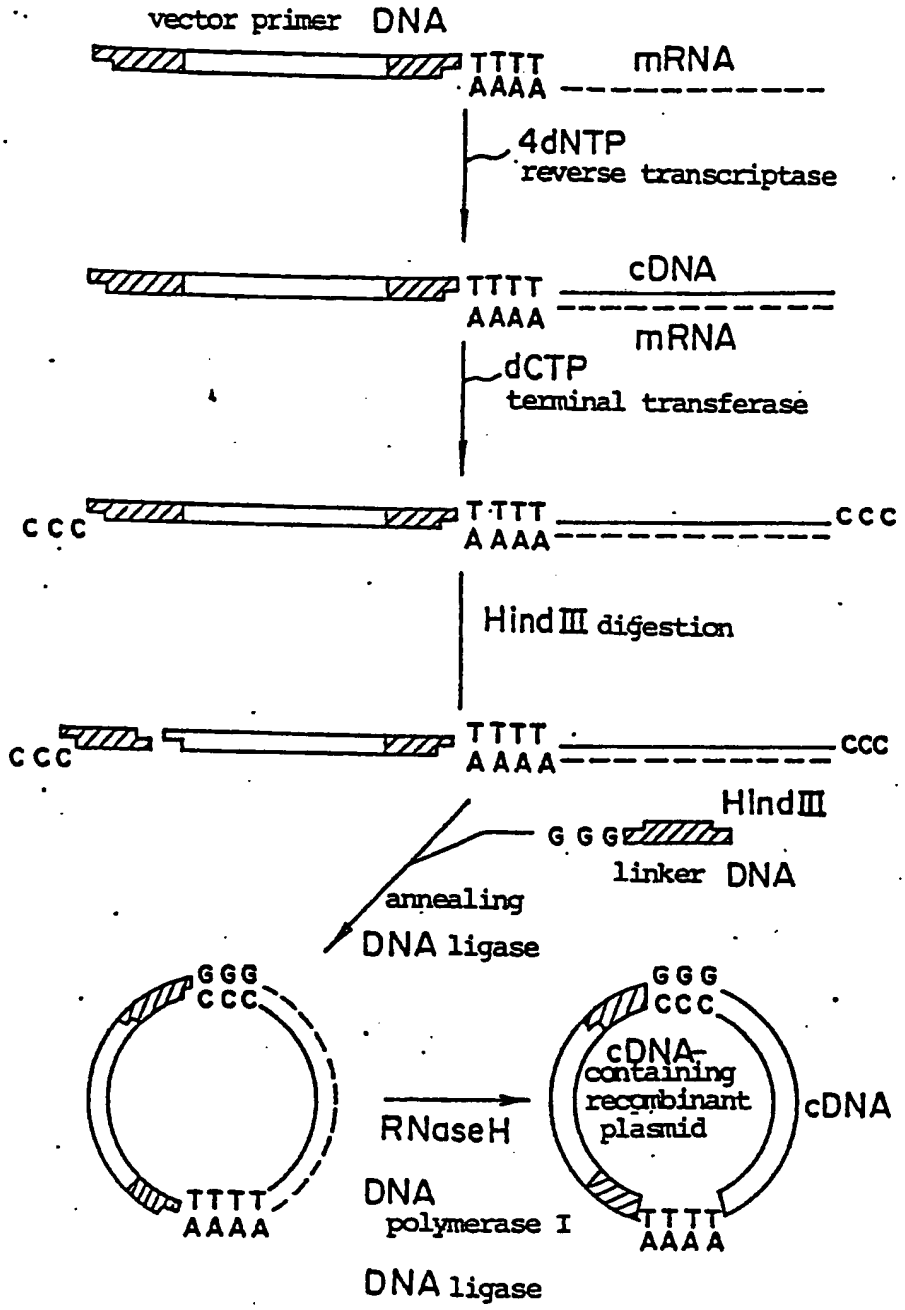

vector primer DNA
TTTT
AAAA
mRNA
4dNTP
reverse transcriptase
cDNA
TTTT
AAAA
mRNA
dCTP
terminal transferase
CCC
TTTT
AAAA
CCC
HindIII digestion
CCC
TTTT
AAAA
CCC
HindIII
G G G
linker DNA
annealing
DNA ligase
GGG
CCC
TTTT
AAAA
RNaseH
DNA
polymerase I
DNA ligase
GGG
CCC
cDNA-containing recombinant plasmid
cDNA
TTTT
AAAA

Fig. 10

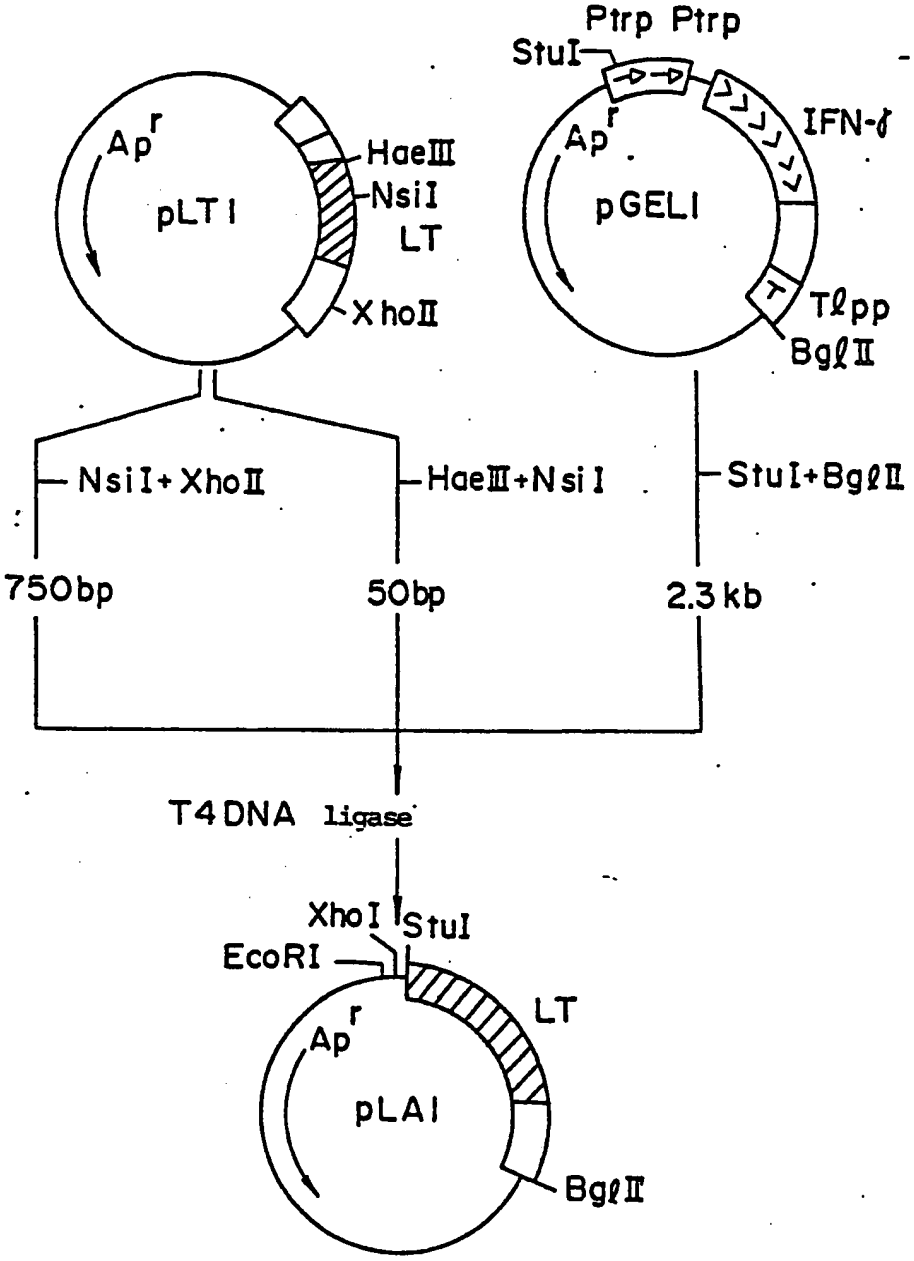

Ptrp Ptrp
StuI
Ap r
IFN-δ
pGELI
Tℓpp
BgℓII
HaeIII
NsiI
LT
pLTI
XhoII
NsiI+XhoII
HaeIII+NsiI
StuI+BgℓII
750bp
50bp
2.3kb
T4DNA ligase
XhoI
StuI
EcoRI
LT
Ap r
pLAI
BgℓII

Fig. 11

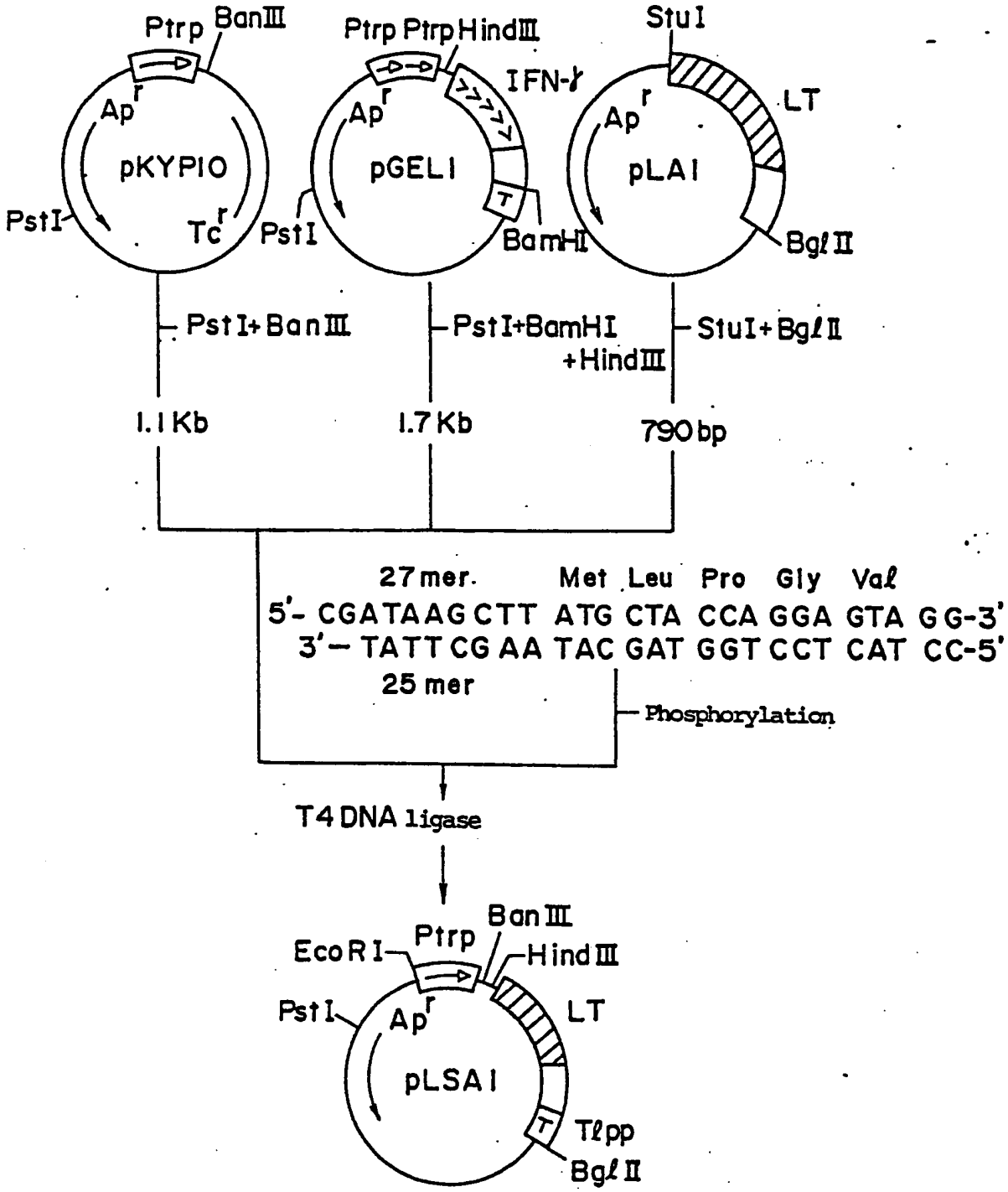

Ptrp
BanIII
Ap r
pKYP10
Tc r
PstI
Ptrp Ptrp HindIII
IFN-γ
Ap r
pGEL1
T
PstI
BamHI
StuI
LT
Ap r
pLA1
BglII
PstI+BanIII
PstI+BamHI
+HindIII
StuI+BglII
1.1Kb
1.7Kb
790bp
27mer
Met Leu Pro Gly Val
5'- CGATAAGCTT ATG CTA CCA GGA GTA GG-3'
3'— TATTCGAA TAC GAT GGT CCT CAT CC-5'
25 mer
Phosphorylation
T4 DNA ligase
BanIII
EcoRI
Ptrp
HindIII
PstI
Ap r
LT
pLSA1
T
Tlpp
BglII

Fig. 12

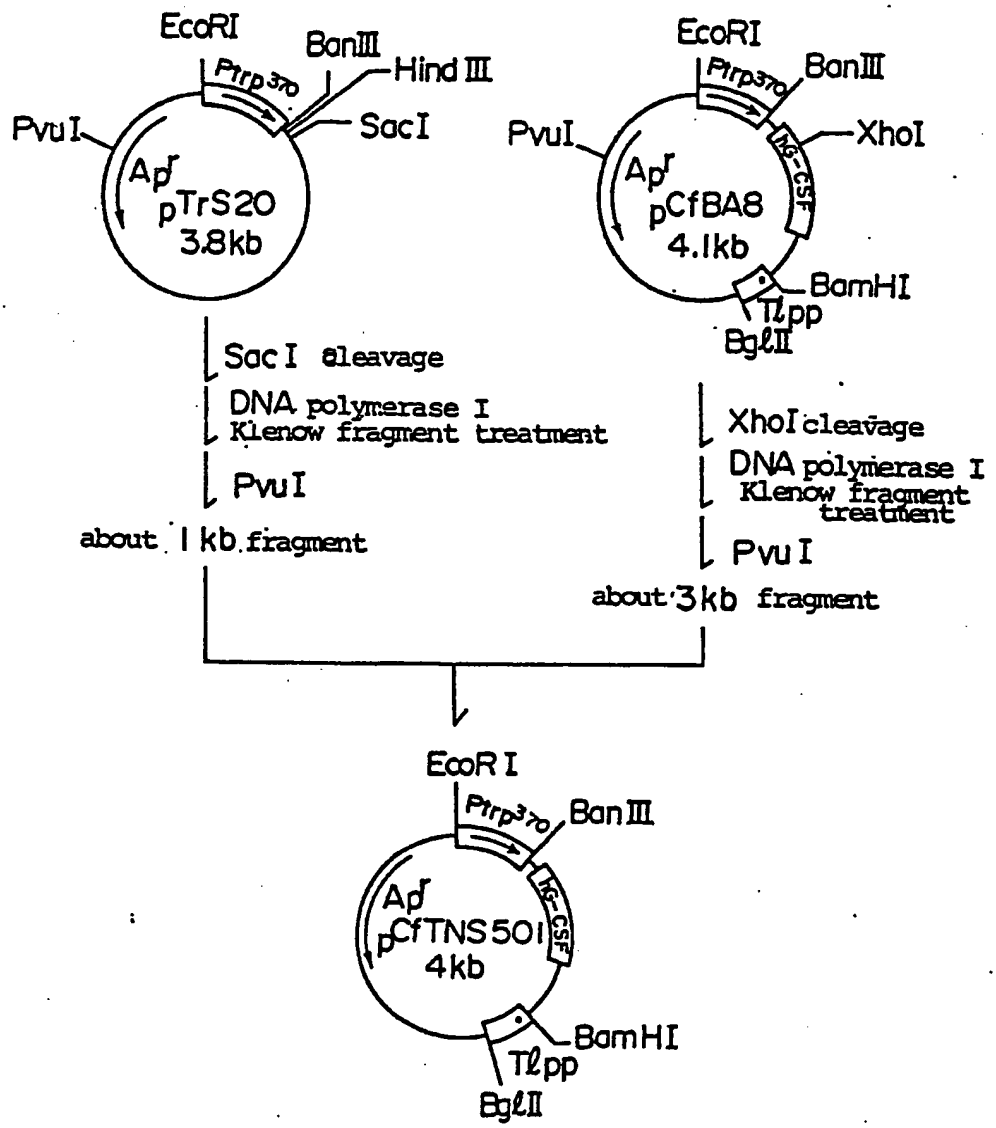

EcoRI
BanIII
Hind III
Ptrp370
PvuI
SacI
Apr
pTrS20
3.8kb
EcoRI
Ptrp370
BanIII
PvuI
XhoI
Ap
pCfBA8
4.1kb
hG-CSF
BamHI
Tlpp
BglII
SacI cleavage
DNA polymerase I
Klenow fragment treatment
PvuI
about 1 kb fragment
XhoI cleavage
DNA polymerase I
Klenow fragment
treatment
PvuI
about 3kb fragment
EcoR I
Ptrp370
BanIII
Ap
pCfTNS501
4kb
hG-CSF
BamHI
Tlpp
BglII

Fig. 13

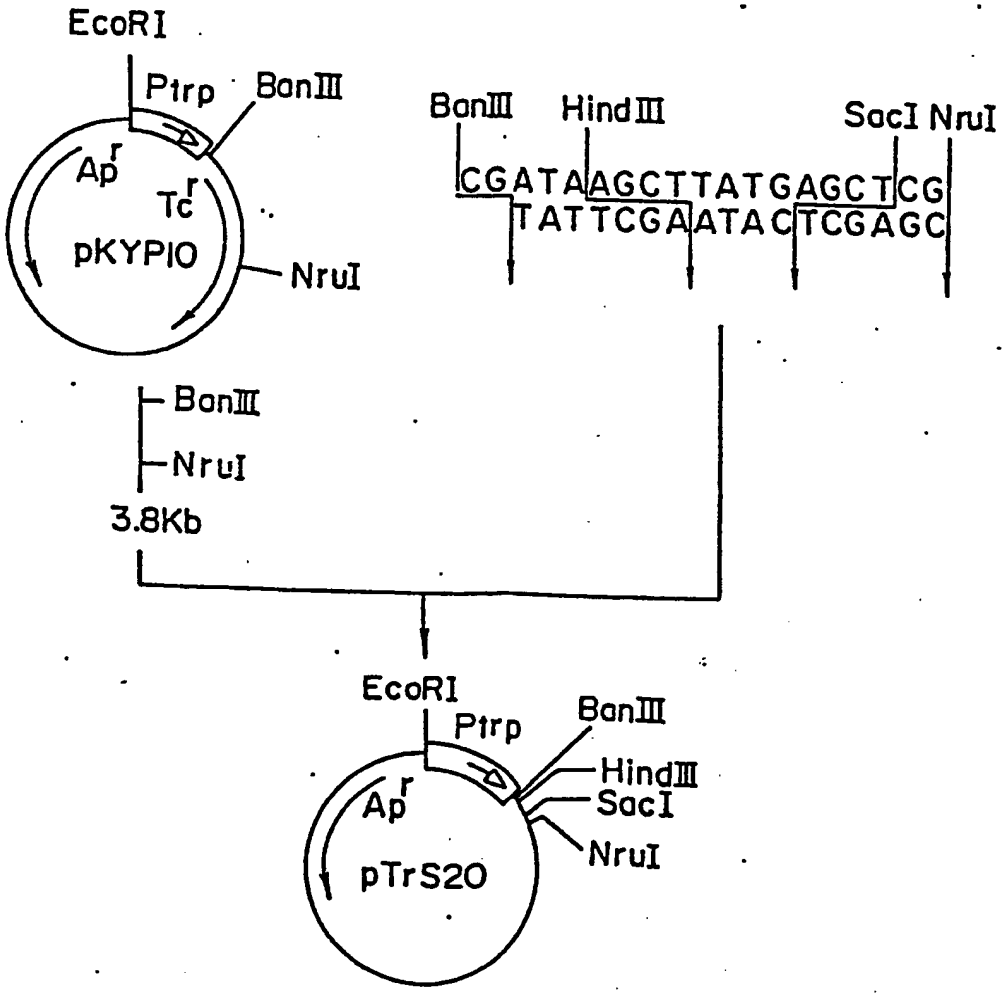

EcoRI
Ptrp
BanIII
Ap^r
Tc^r
pKYP10
NruI
BanIII
NruI
3.8Kb
BanIII
HindIII
SacI
NruI
CGATAAGCTTATGAGCTCG
TATTCGAATACTCGAGC
EcoRI
Ptrp
BanIII
HindIII
SacI
NruI
Ap^r
pTrS20

Fig. 14

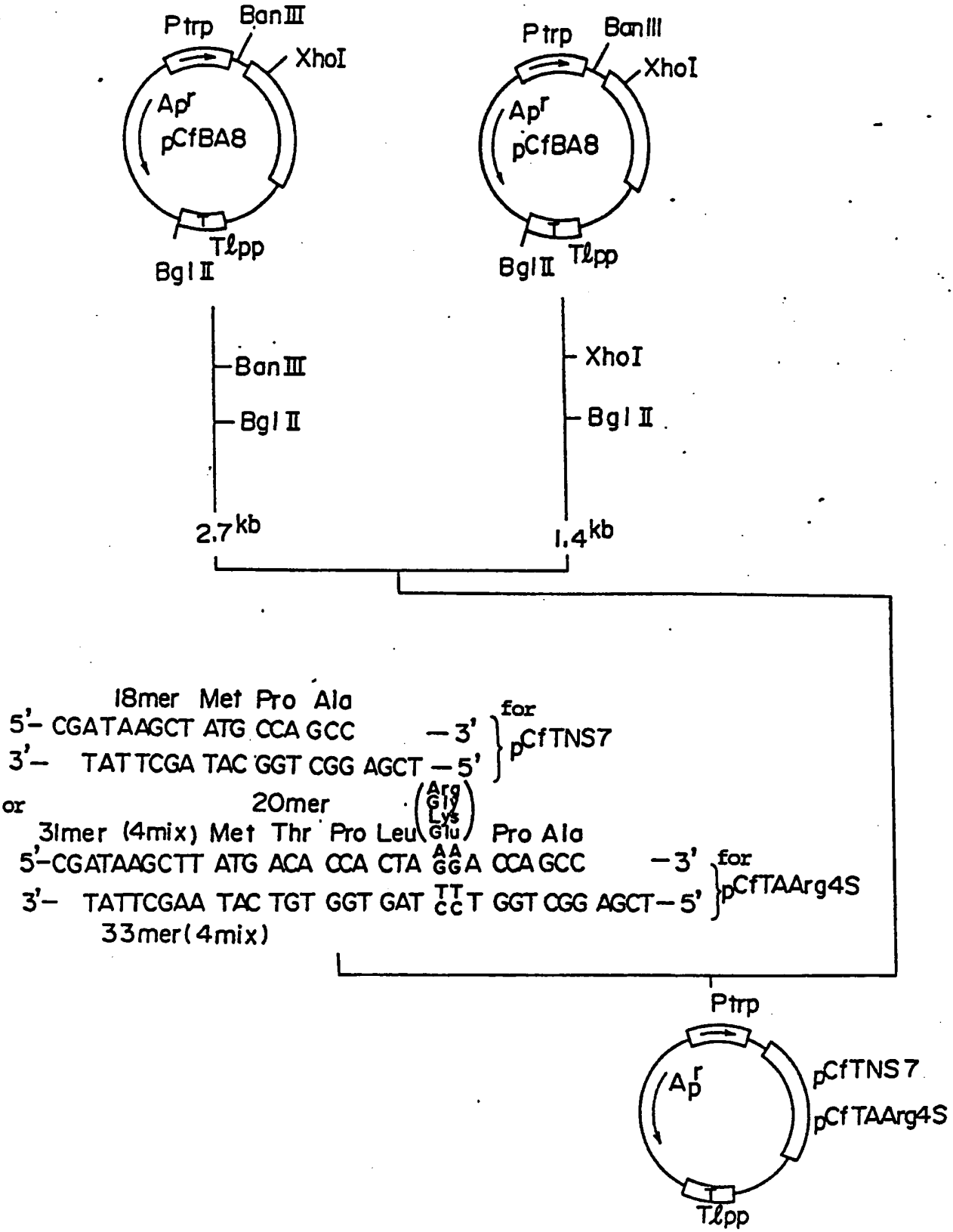

Ptrp
BanIII
XhoI
Apr
pCfBA8
Tlpp
BglII
BanIII
BglII
2.7kb
XhoI
BglII
1.4kb
18mer Met Pro Ala
5'- CGATAAGCT ATG CCA GCC -3'
3'- TAT TCGA TAC GGT CGG AGCT -5'
for pCfTNS7
or
20mer
(Arg Gly Lys Glu)
31mer (4mix) Met Thr Pro Leu Pro Ala
5'-CGATAAGCTT ATG ACA CCA CTA AA GGA CCA GCC -3'
3'- TATTCGAA TAC TGT GGT GAT TT CC T GGT CGG AGCT-5'
33mer(4mix)
for pCfTAArg4S
Ptrp
Apr
pCfTNS7
pCfTAArg4S
Tlpp

Fig. 15

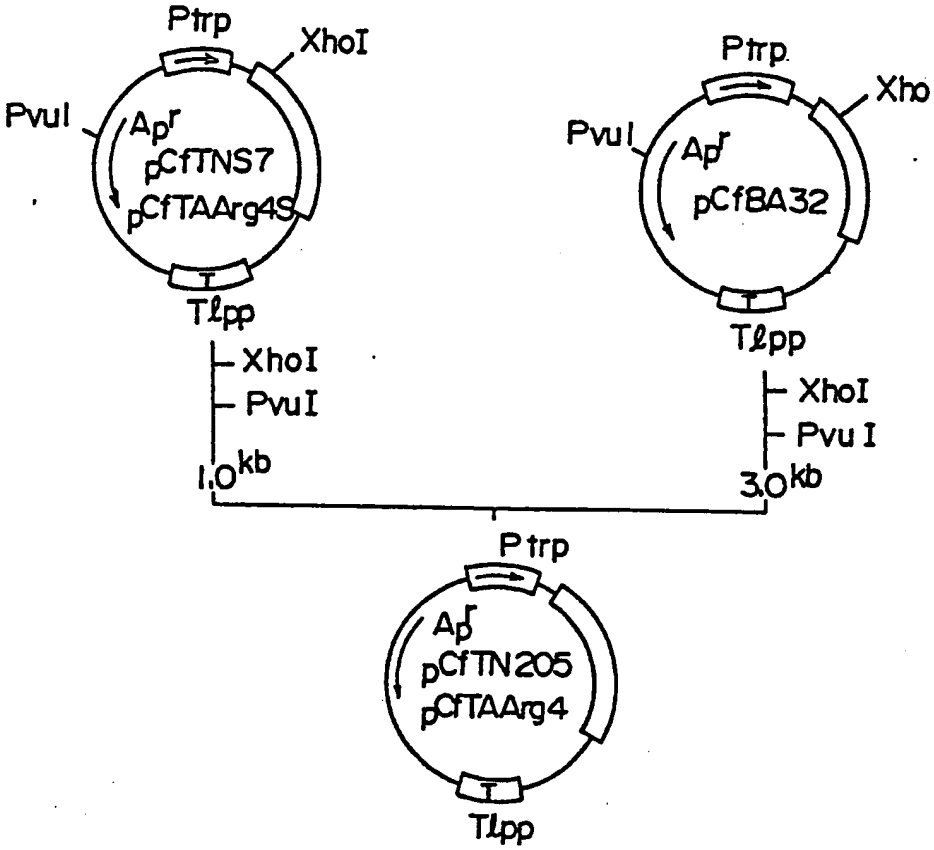

Ptrp
XhoI
PvuI
Ap^r
pCfTNS7
pCfTAArg4S
T
Tlpp
XhoI
PvuI
1.0kb
Ptrp
Xho
PvuI
Ap^r
pCfBA32
T
Tlpp
XhoI
Pvu I
3.0kb
Ptrp
Ap^r
pCfTN205
pCfTAArg4
T
Tlpp

Fig. 16

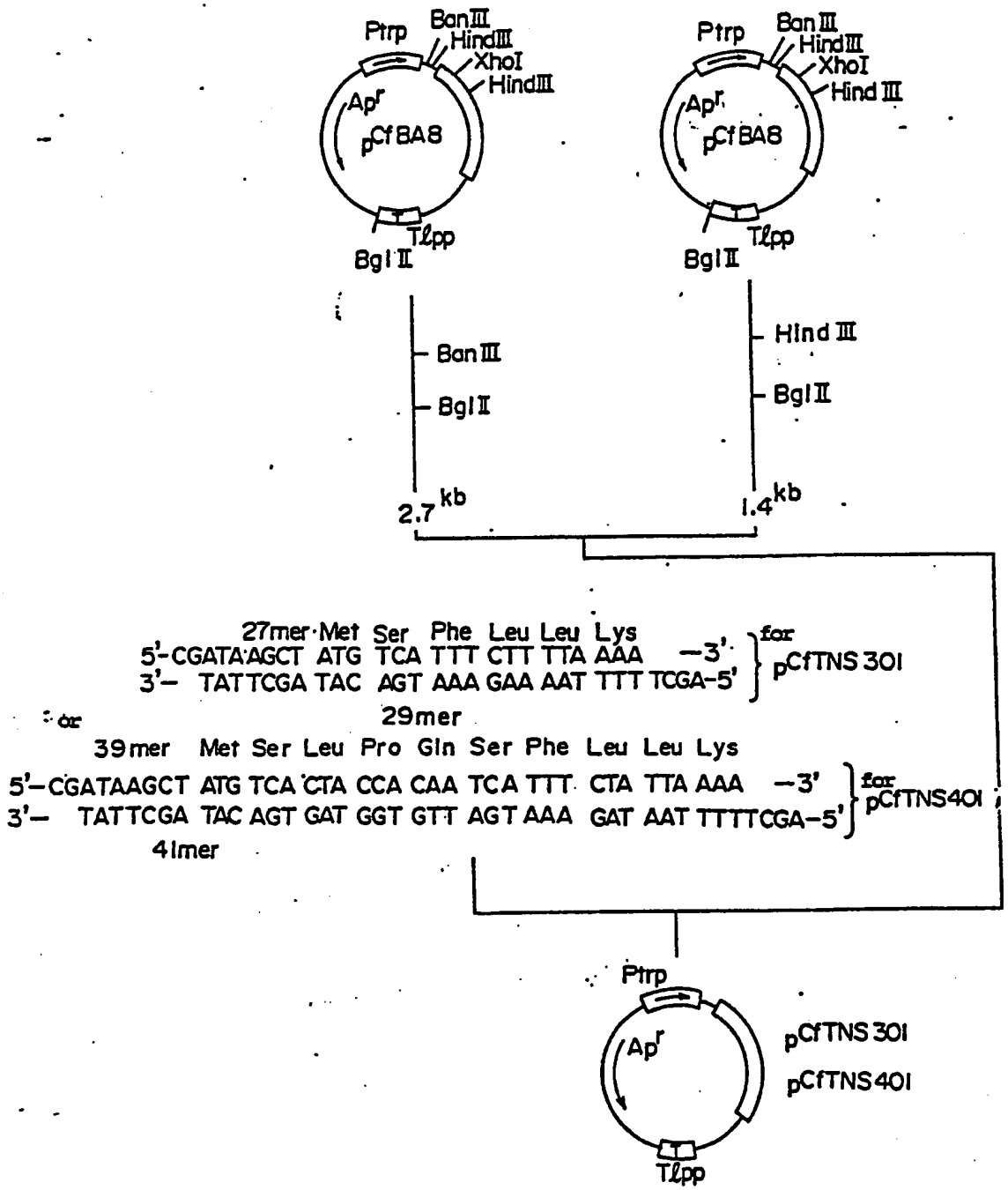

Ptrp
BanIII
HindIII
XhoI
HindIII
Ap^r
pCfBA8
Tlpp
BglII
BanIII
BglII
2.7 kb
HindIII
BglII
1.4 kb
27mer Met Ser Phe Leu Leu Lys
5'-CGATAAGCT ATG TCA TTT CTT TTA AAA —3'
3'— TATTCGA TAC AGT AAA GAA AAT TTT TCGA-5'
for pCfTNS301
or
29mer
39mer Met Ser Leu Pro Gln Ser Phe Leu Leu Lys
5'—CGATAAGCT ATG TCA CTA CCA CAA TCA TTT CTA TTA AAA —3'
3'— TATTCGA TAC AGT GAT GGT GTT AGT AAA GAT AAT TTTTCGA—5'
for pCfTNS401
41mer
Ptrp
Ap^r
Tlpp
pCfTNS301
pCfTNS401

Fig. 17 (1)

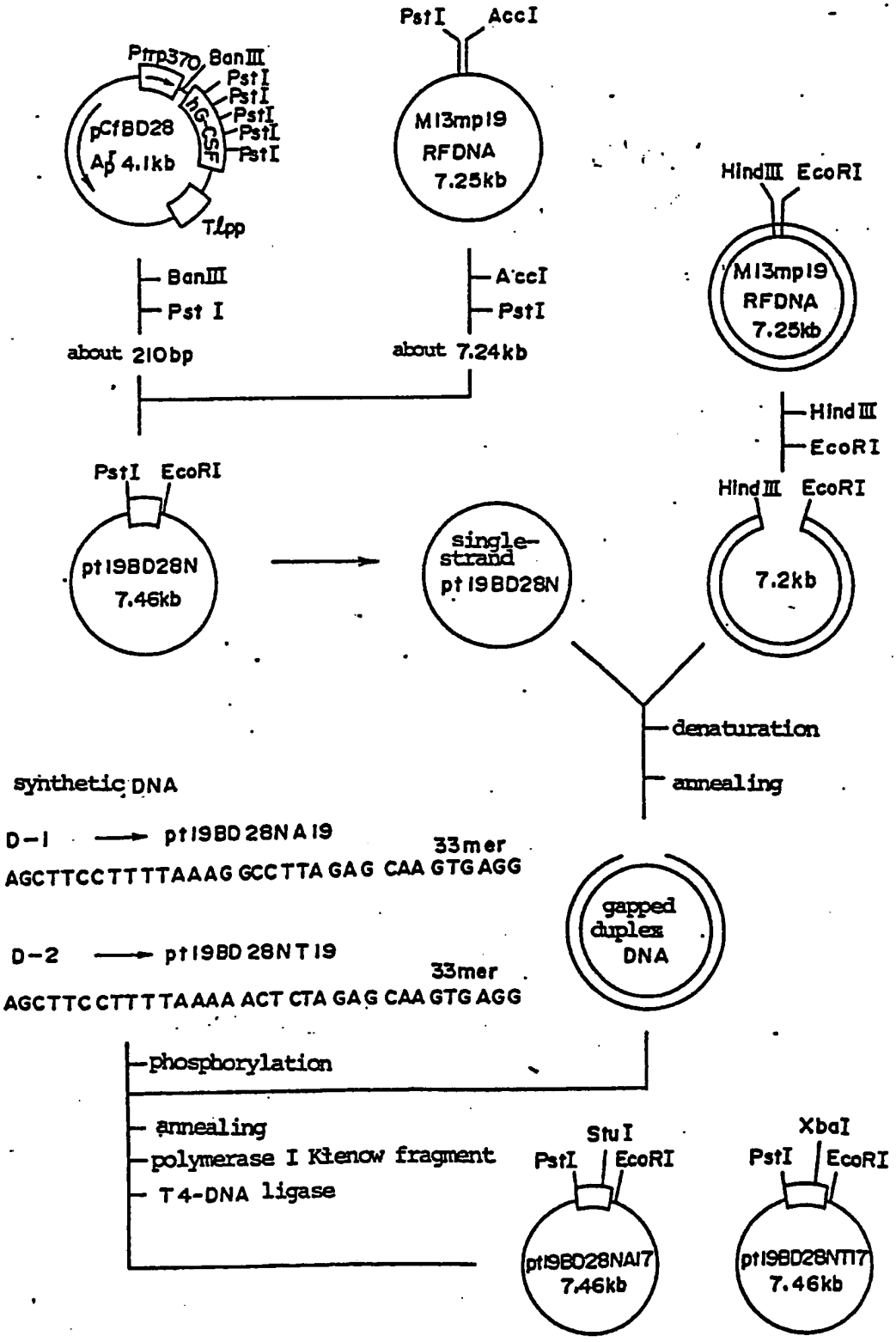
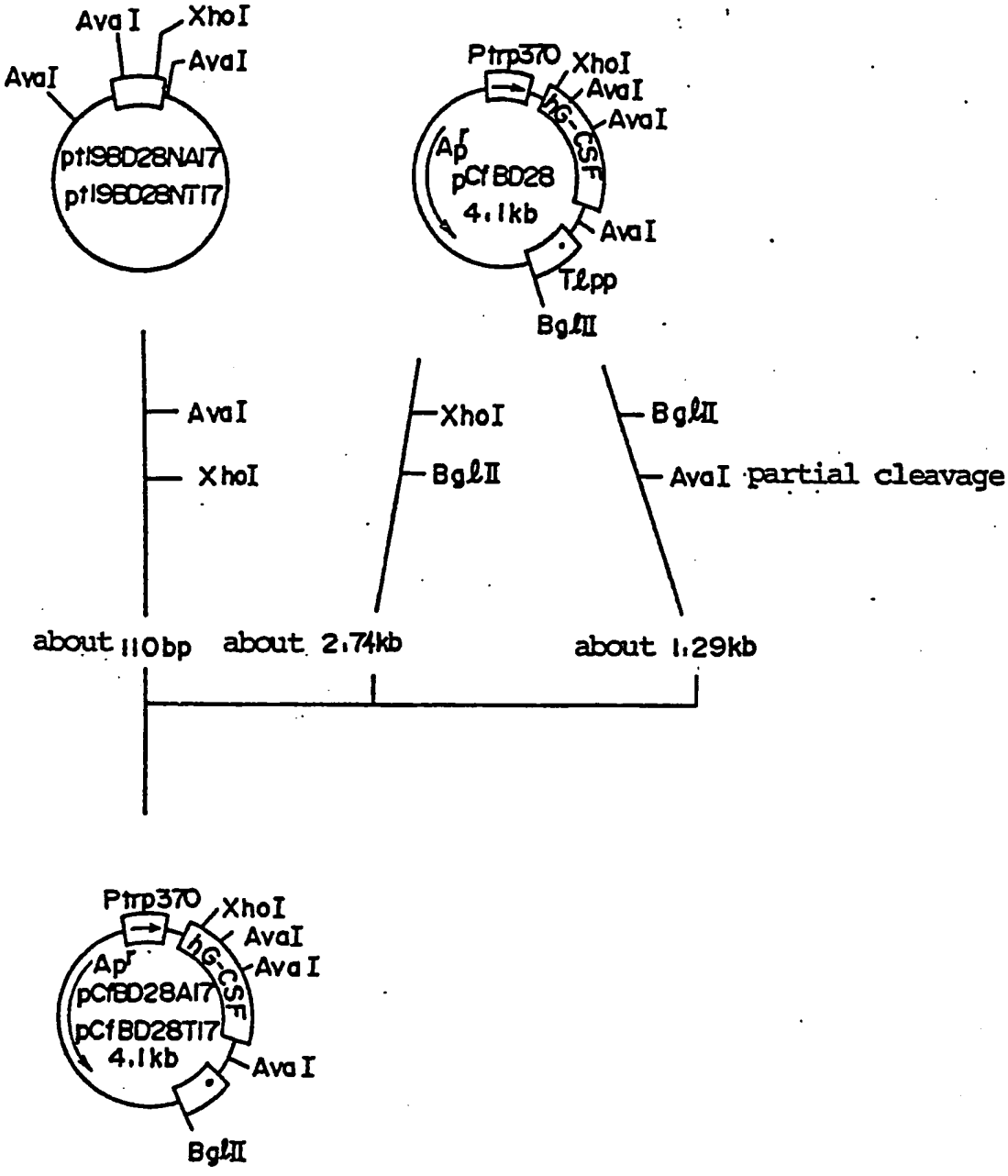

PstI
AccI
Ptrp370
BanIII
PstI
PstI
PstI
PstI
PstI
hG-CSF
pCfBD28
Aβ 4.1kb
Tlpp
M13mp19
RFDNA
7.25kb
HindIII
EcoRI
M13mp19
RFDNA
7.25kb
BanIII
Pst I
about 210bp
AccI
PstI
about 7.24kb
HindIII
EcoRI
HindIII
EcoRI
PstI
EcoRI
ptI9BD28N
7.46kb
single-strand
ptI9BD28N
7.2kb
denaturation
annealing
synthetic DNA
D-1 → ptI9BD28NA19
33mer
AGCTTCCTTTTAAAG GCCTTA GAG CAA GTG AGG
D-2 → ptI9BD28NTI9
33mer
AGCTTC CTTTTAAAA ACT CTA GAG CAA GTG AGG
gapped duplex DNA
phosphorylation
annealing
polymerase I Klenow fragment
T4-DNA ligase
StuI
PstI
EcoRI
XbaI
PstI
EcoRI
ptI9BD28NAI7
7.46kb
ptI9BD28NTI7
7.46kb

Fig. 17 (2)


AvaI
XhoI
AvaI
AvaI
pt19BD28NAI7
pt19BD28NTI7
Ptrp370
XhoI
AvaI
AvaI
hG-CSF
Ap
pCfBD28
4.1kb
AvaI
Tlpp
BglII
AvaI
XhoI
XhoI
BglII
BglII
AvaI partial cleavage
about 110bp
about 2.74kb
about 1.29kb
Ptrp370
XhoI
AvaI
AvaI
hG-CSF
Ap
pCfBD28AI7
pCfBD28TI7
4.1kb
AvaI
BglII

European Patent Office

EUROPEAN SEARCH REPORT

Application number

DOCUMENTS CONSIDERED TO BE RELEVANT

EP 87119157.3

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	EP - A1 - 0 169 566 (CHUGAI SEIYAKU KABUSHIKI KAISHA) * Page 20, lines 15,16 * --	1-4,16, 27	C 12 N 15/00 C 07 K 15/00 C 07 H 21/04 C 12 N 1/20 C 12 P 21/00 //(C 12 N 1:20, C 12 R 1:19)
P,X	WO - A1 - 87/01 132 (KIRIN-AMGEN, INC.) * Claims 1,7,38,42 * --	1-6,8, 27	
A,P	EP - A1 - 0 220 520 (CHUGAI SEIYAKU KABUSHIKI KAISHA) * Abstract; fig. 5; claims * --	1,5,6, 10,13	
A	WO - A1 - 86/04 605 (CHUGAI SEIYAKU KABUSHIKI KAISHA) * Abstract * --	1,5,6, 10,13	
D,A	NATURE, vol. 319, 30 January 1986, (New York, London) S. NAGATA et al. "Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor" Pages 415-418 * Totality * --	1	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 12 N
C 07 K
C 07 H
C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	31-03-1988	WOLF

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
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L : document cited for other reasons

& : member of the same patent family, corresponding document

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DOCUMENTS CONSIDERED TO BE RELEVANT

EP 87119157.3

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,A	SCIENCE, vol. 232, no. 4746, 4 April 1986 (Washington DC) L.M. SOUZA et al.: "Recombinant Human Granulocyte Colony-Stimulating Factor: Effects on Normal and Leukemic Myeloid Cells" Pages 61-65 * Totality * ----	1	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	31-03-1988	WOLF

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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